# STUDIES ON SYNTHETIC AND NATURALLY OCCURRING ENZYME METABOLITES

THESIS

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PREFACE.

The work described in this dissertation was carried out in the Agricultural Section of the Chemistry Department, Glasgow University, under the supervision of Dr.W.R.Rees, from October 1958 to June 1963.

I would like to thank Dr.W.R.Rees, who initiated this work, for his constant encouragement and help throughout its course. Acknowledgements for gifts of material and for help at various stages of the work are made in the main text.

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## ABBREVIATIONS USED.

| | | |
|---|---|---|
| RNA | ------- | ribonucleic acid. |
| DNA | ------- | deoxyribonucleic acid. |
| XMP | ------- | mixed 2'-and 3'-phosphate esters of nucleoside containing base X. |
| X5'P | ------- | 5'-phosphate ester of nucleoside containing base X. |
| XDP | ------- | 5'-pyrophosphate of nucleoside containing base X. |
| XTP. | ------- | 5'-triphosphate of nucleoside containing Base X. |
| polyX | ------- | polyribotide containing single base X. |
| PRP | ------- | polyribose phosphate. |
| R5P | ------- | ribose-5-phosphate. |
| R5PP | ------- | ribose-5-pyrophosphate. |
| TMV | ------- | Tobacco mosaic virus. |
| PAP | ------- | adenosine-2(3');5'-diphosphate. |

## GENERAL INTRODUCTION.

### Ribonucleic Acid; its occurrence, structure and functions.

The recognition of DNA as the carrier of genetic information and the discovery that this information is expressed in the synthesis of proteins by the mediacy of RNA, has given impetus to studies of these materials in many fields of investigation, and in the past decade this intensive research has been rewarded by discoveries of the highest importance.

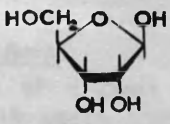
In contrast to the present rapid expansion of the subject, progress during the eighty years subsequent to the discovery of the nucleic acids by Miescher was slow. Miescher isolated the material in 1868 - 69 by acid precipitation, from the nuclei of pus cells and later from salmon sperm heads, and recognised its high phosphorus and nitrogen content and its acidic nature (1). By the early nineteen hundreds other workers had isolated nitrogenous bases from the products of acid hydrolysis and identified them as purines and pyrimidines (2,3,4,5). Two purines, adenine and guanine and three pyrimidines, uracil, cytosine and thymine were found as nucleic acid components and in addition Hammarsten demonstrated the presence of a

carbohydrate (6). In identifying the carbohydrate components of "yeast" nucleic acid (7) and "thymus" nucleic acid as ribose and deoxyribose respectively Levene firmly established the existence of two distinct classes of nucleic acid and provided the basis for nomenclature which is still used. Furthermore, it was found that although the bases guanine, adenine and cytosine are common to both types of nucleic acid, uracil is unique to "yeast" nucleic acid (ribonucleic acid - RNA) and thymine to "thymus" nucleic acid (deoxyribonucleic acid - DNA).

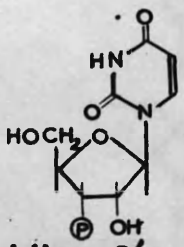
When ribonucleic acid is degraded by alkali, products which contain a single base, a phosphate group, and ribose in equimolar proportions are obtained. These are the nucleotides. Mild acid hydrolysis of the nucleotides removes the phosphate groups to form nucleosides, which are N-ribosides of the bases. In these compounds purine bases are attached to the sugar at the 9 nitrogen in the imidazole ring (9) and the pyrimidines at the 3 nitrogen atom (10). The furanose configuration of the ribose was proved by comparing the trimethyl ribose derivatives obtained by methylating the nucleosides and subsequently removing the base, with synthetic 2,3,5 - trimethyl-D-ribofuranose (11).

FIG. 1

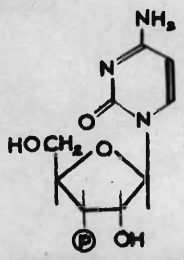
Structures of the common constituents of RNA



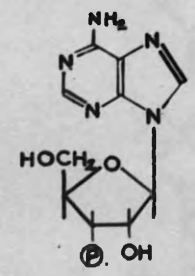
β-D-Ribofuranose



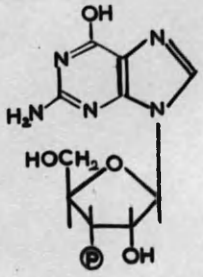
uridine-3′-phosphate



cytidine-3′-phosphate

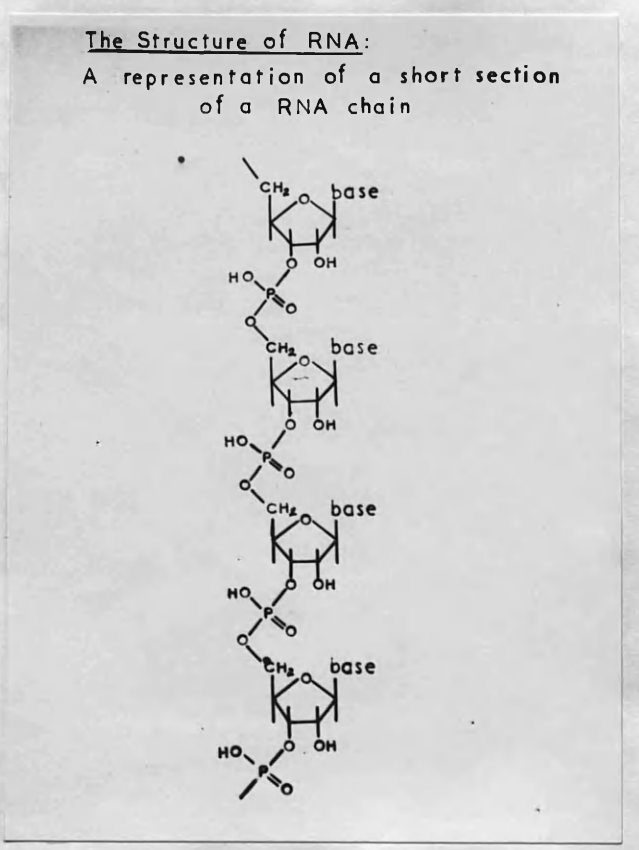


adenosine-3′-phosphate



guanosine-3′-phosphate

FIG. 2.
Structure of Ribonucleic acid.



Later, proof was obtained that the glycosidic linkage is in the $\beta$-configuration(12). In 1926 Levene and Simms (13) from the results of electrometric titration of mono-nucleotides and nucleic acids proposed a structure for the nucleic acids in which the sugar moieties of the nucleosides are joined through phosphodiester linkages. However, the important question as to which hydroxyl groups of the ribose moieties are involved in ester formation was not settled until 1953. Proof that the 3'-hydroxyl group is involved was afforded by the demonstration (14) that pancreatic ribonuclease is specific for phosphodiesters at this position. The discovery that 5'-phosphate esters are obtained by the action of snake venom diesterase (15) showed that the other position of attachment is the 5'-hydroxyl group. Earlier evidence for a 5' linkage (16) based on rather inconclusive enzyme work was not widely accepted because of the failure to obtain the 5'-nucleotides by chemical hydrolysis; Brown and Todd, however, have proposed a mechanism (17) which accounts for the formation of 2' and 3'-phosphomonoesters by hydrolysis of 3'-5' linkages (See Appendix).

Thus the structure of ribonucleic acid which emerged after more than eighty years of continuous research can be briefly outlined as follows. The basis of the

structure is a polymeric backbone of ribofuranose units linked through the 3 and 5 positions by phosphodiester bridges. Each ribose moiety is joined by a $\beta$-glycosidic linkage to one of 4 nitrogeneous bases. This structure which, with some augmentations, is still widely accepted is illustrated in Fig. 2. The existence of chain branching, though not completely excluded, is now thought improbable. The only sugar, other than ribose, to have been found in ribonucleic acid is 2-O-methylribose, which is a minor constituent of "soluble" RNA. This type of RNA also contains unusual bases ( in particular methylated derivatives of the four common bases). These are discussed in a later section.

Once the basic structure had been established investigation into more subtle details gained priority. Attempts to distinguish differences in the ribonucleic acids of various sources by determining their base composition met with partial success; for instance it was shown that the RNAs of different strains of tobacco mosaic virus are identical in base composition but differ from the closely related cucumber viruses (18). However, this approach to the analysis of the nucleic acids of more complex organisms was made obsolete by the discovery that the cell contains a complex population of RNAs rather than a single molecular

species.The first indications of heterogeneity arose from observations of the location of RNA in the cell. Cytological techniques including ultraviolet cytophotometry (19), staining methods (20), the use of specific enzymes (20) and more recently electron microscopy (21) have shown that a major proportion of the RNA is associated with granules which are located in the ground cytoplasm. These granules also contain a basic protein and consequently were called ribonucleoprotein particles (RNP). They are found in cells of all types. In highly organised cells which contain a well defined nucleus, a body known as the nucleolus, which is closely associated with the nucleus, is found to contain a very high concentration of RNA (22). This work was extended when improved techniques of centrifugation (24) made possible the fractionation of disrupted cell preparation into well defined subfractions, which can be isolated in quantity sufficient for analysis. Thus it was shown that 80-90% of the total RNA of the cell is contained in particles which are sedimented between 6,000 and 100,000 g. (25). These are the cytoplasmic granules which had been described by the cytologists and it was found that in addition to RNA, these particles, now commonly referred to as ribosomes, contain a basic protein (26), the polyamines putrescine and spermidine (27) and magnesium (25). In

homogenates of mammalian cells, the ribosomes are organised into larger units known as microsomes by a lipoprotein membrane and it appears that the membranous component is a fragment of the endoplasmic reticulum to which the ribosomes are attached in the cell (28). It is usual to refer to the ribosomes or microsomes as the particulate fraction.

The cell sap, which remains after removing the particulate fraction, contains 10-20% of the total RNA. This fraction is apparently unassociated with protein and remains in solution after centrifugation for 2 hrs. at 100,000 g. For this reason it is often referred to as soluble RNA (sRNA). From differences in physical properties and base composition it has been established that sRNA and ribosomal RNA are distinct molecular types; these properties are described in a later section. The most exciting development in this regard however, has been the assignment of unique biological roles to the particulate and soluble fractions.

It had long been suspected that RNA has some connection with protein synthesis. Measurement of the concentration of RNA in different materials had shown that

its concentration is highest in cells which are actively reproducing (29) or which have a high turnover of protein (19,3 When the advent of isotopically labeled amino-acids presented the opportunity to test their incorporation by subcellular fractions, it was quickly established that the ribosomes provide the sites at which amino-acids are assembled into polypeptides (31). Simultaneously it was demonstrated that a soluble enzyme fraction and ATP were essential for the incorporation (32). The soluble fraction can be precipitated by adjusting to pH5 and is often referred to as the pH 5 fraction. In addition to protein the pH 5 fraction contains the soluble RNA previously described and when it was shown that incubation with amino-acids in the presence of ATP results in combination of the amino-acids with sRNA molecules, it became clear that the sRNA plays an important part in the incorporation of the amino-acids at the ribosomes. Some details of the process by which the amino-acids are combined with the sRNA - so-called amino-acid activation - are now known. Each sRNA molecule is terminated by an adenosine moeity and the amino-acid becomes attached at the 2'- or 3'-hydroxyl group of this adenosine through an ester linkage (32). Previously it had been shown that the non-particulate

fraction contains enzymes which catalyse the formation of enzyme bound amino-acyl adenylate, from aminoacids and ATP, with the elimination of inorganic pyrophosphate (33). It is now known that there exists for each amino acid, a unique combination of sRNA molecule + activating enzyme, and it appears likely that it is at this stage - transfer of amino-acid to sRNA - that "mistakes" in the final amino-acid sequence of the protein are avoided (34). The reactions leading to the formation of amino-acyl RNA can be formulated as follows:

$$\text{amino-acid} + \text{ATP} + \text{enzyme} \rightarrow \underbrace{\text{amino-acyladenylate}}_{\text{enzyme}} + PP_i$$

$$\underbrace{\text{amino-acyladenylate}}_{\text{enzyme}} + \text{sRNA} \rightarrow \text{amino-acyl RNA} + \text{AMP} + \text{enzyme}$$

The particular hydroxyl group involved in ester formation is not yet known. For each amino-acid,

one enzyme catalyses both condensation with ATP and transfer to sRNA.

The next step in protein formation is the transfer of the amino-acid, still attached to its molecule of sRNA, to the ribosomes. This process is often referred to as amino-acid transfer and from this function of sRNA the name transfer RNA has derived. Also in current usage are the terms amino-acid acceptor RNA and shuttle RNA. In this discussion the fraction as a whole will be referred to as sRNA and specific molecules by prefixing the name of the amino-acid to "transfer RNA": thus for instance, the sRNA specific for valine is valyl-transfer RNA.

It is proposed that some interaction between the bases of the sRNA bearing amino-acids and the ribosomal RNA aligns the amino-acids in a position favourable to condensation. This model, known as the adaptor hypothesis (35) also suggests a means by which the sequence of amino-acids in the polypeptide chain, can be determined by the sequence of the bases in a polynucleotide chain.

The adaptor hypothesis was first proposed in

1955 and at that time a great deal of evidence had accumulated which implicated DNA as the carrier of genetic information and indicated that its role in directing the synthesis of species specific proteins is an indirect one. This evidence has been summarised in a review by Berg (36). It was well established that the "information" for the amino-acid sequence of a particular protein lies in the base sequence of a corresponding stretch of the DNA of the nucleus (the genome or cistron) and it was postulated that the "information" is transferred to the base sequence of the ribosomal RNA when this is synthesised in the nucleus. The relation of the base sequence in the DNA and RNA to the amino-acid sequence in the protein is clearly not a simple one, since there are only four bases and approximately 20 amino-acids. The relationship is referred to as the genetic code and the adaptor hypothesis was an attempt to provide a mechanism by which the base sequence information could be translated into an amino-acid sequence. The hypothesis was presented as a coding mechanism on theoretical grounds before the discovery of the function of soluble RNA. Its author, F.H.C. Crick starting from the assumption that the amino-acid sequence in proteins is determined by the base sequence in a polynucleotide,

argued (35) that the physico-chemical properties of the amino-acids and the bases were incompatible with a direct interaction between them. He proposed that before interaction with the polynucleotide template could occur, the amino-acid must combine with some adaptor molecule, specific for each amino-acid, which was able to recognise or become adapted to a site on the template by an interaction which involves hydrogen bonding. This brilliant prediction was rapidly followed by the discovery of the mechanism of amino-acid activation, and the sRNA was seen to fill the adaptor requirement.

The hypothesis requires that there shall be a unique sRNA molecule for each amino-acid and that the template polynucleotide shall contain sequences of bases which are complementary to those of the sRNA molecule which define the amino-acid it carries.

As has been stated above it was once thought that the coded information for protein synthesis lay permanently in the base sequence of ribosomal RNA (i.e. that ribosomal RNA provides the "template" polynucleotide), but a body of indirect evidence accumulated which indicated that the

templates were in fact provided by a different type of RNA (36,37). On the basis of experiments with enzyme deficient bacterial mutants, Jacob and Monod (38) proposed that there is a species of RNA whose function is to carry the genetic information from the nucleus to the ribosomes. They proposed the name messenger RNA (others have used informational RNA and template RNA) for this fraction. Evidence for its existence has now been obtained from a wide variety of experiments. It is particularly easy to identify in virus infected cells and in fact it was recognised in phage infected bacteria as a type distinct from ribosomal and sRNA long before the tentative assignment of its biological role. Volkin and Astrachan were the first to demonstrate (39) that immediately following the invasion of E. Coli by phage, there occurs a rapid synthesis of a new type of RNA and that the base composition of this RNA mimics that of the phage DNA (39,40). Evidence that the base sequence of the RNA is complementary to the phage DNA was obtained by the formation of stable hydrogen-bonded hybrids (41). Similar evidence indicates the presence of a small fraction (approximately 3%) among the RNAs of normal cells which bears a complementary relationship to its homologous DNA (42). Other charac teristic

of this RNA which support the proposed role are its rapid turnover, the heterogeneity of its molecular weight, and its ability to form complexes with ribosomes (43).

The existence of a RNA which carries information from the nucleus to the ribosomes would not invalidate the basic mechanism for protein formation proposed in the adaptor hypothesis. Crick originally proposed that the template is the ribosomal RNA; messenger RNA could equally well fill this role.

Recent experiments which demonstrated that a cell free system from E.Coli is stimulated into synthesising polyphenylalanine when poly U is added to the system (44) support the view that amino-acid sequence is directed by a ribopolynucleotide. The incorporation of other amino-acids is stimulated by synthetic polynucleotides of different base composition and experiments with this system have formed the basis of attempts to solve the amino-acid code (45).

An elegant experiment with this system has served to demonstrate the specificity of sRNA - template RNA interaction and has thus confirmed the adaptor hypothesis.

The synthetic polynucleotide poly UG stimulates the incorporation of cysteine into polypeptides when the cysteinyl -transfer RNA -cysteine (i.e. sRNA bearing molecule of cysteine) complex is added to the system. Now when cysteinyl - RNA - cysteine is reduced by catalytic hydrogenation to cysteinyl - RNA - alanine, the same system with poly UG is able to incorporate alanine (46). This experiment proved that the position of the amino-acid in the polypeptide is determined not by the amino-acid, but by the sRNA molecule which carries it.

Thus it appears that ribonucleic acids have three functions in protein synthesis. The sRNA molecules carry the amino-acids to the ribosomes and by specific interaction with a template determine the final position of the amino-acids in the protein. It is probable that the sRNA molecules are also concerned in the condensation reaction. Messenger RNA, it is proposed, is synthesised at the nucleus with its base sequence complementary to that of a cistron and is then transferred to the cytoplasm where it acts, at the ribosomes, as a template for protein synthesis. The specific function of ribosomal RNA is as yet obscure.

The recognition of their intimate association with protein synthesis stimulated intensive efforts to discover more detail in the structures of the various RNA fractions in order to relate their structures to their functions.

Little is known about the messenger RNA fraction beyond what has already been described, that is beyond the properties which serve to distinguish it from sRNA and ribosomal RNA and which also serve as evidence for its proposed function (43). Owing to its low concentration, heterogeneity, and metabolic instability, this fraction is more difficult to isolate than the other fractions. In addition, ribosomal and sRNA have been recognised as distinct fractions for a longer period and consequently have been subject to more prolonged study. The properties of these two fractions are described in the following paragraphs and where this is possible, structural features related to biological functions are noted.

Although sRNA and ribosomal RNA are easily separated from each other, in the ultracentrifuge, isolation of single molecular species from either group

is difficult. Fractionation and other operations on RNAs are hindered by the fragile nature of the phosphodiester linkage and also by the fact that each group contains a complex mixture of molecules with very similar properties. Consequently, with few exceptions, studies have been carried out on samples which have not been fractionated beyond the separation of the sRNA and ribosomal RNA. Nevertheless, valuable information regarding the differences between these two fractions have been obtained and specific properties have been associated with each of them.

The structure and properties of soluble RNA.

Of the three categories of RNA which have been described, soluble RNA has proved most amenable to structural studies. This may be attributed to two properties unique to this fraction. First its low and homogeneous molecular weight and its apparent freedom from association with protein, permits an easy isolation of the population of sRNA molecules as a whole. Secondly, its unique ability of combining with amino-acids has been exploited as a means of assaying specific sRNA molecules and has aided in their isolation. The work of Zamecnik and his colleagues provides an elegant example of the application of these methods (62).

Estimates of the molecular weight of this fraction by ultracentrifugal analysis (48,49,50,51) and end group analysis (52,53) agree that the chain length is between 70 and 90 nucleotides.

It was mentioned previously that sRNAs have in common an adenosine moiety at the amino-acid acceptor end. In fact, it seems that similarity extends beyond this position and that the common grouping at the amino-acid acceptor end is (54)

adenylyl-5'-3'cytidylyl-5'-3'cytidylyl-5'-3'RNA

more usually abbreviated thus RNA.....pCpCpA.

At the non-acceptor end, each chain is terminated by a guanosine-5'-phosphate residue (55).

Observations of the base composition of sRNAs (53,56) from a variety of sources have revealed two outstanding features which are common to all of them. The presence of a relatively high fraction of the "unusual" nucleotides such as ribothymidylate, 2-methyladenylate, 6-methyl-aminopurine nucleotide, 1-methylguanylate in proportions of

0.07 to 1% of the total bases is a unique feature of the sRNA fraction. Cantoni et al. have recently suggested (57) that the sRNA specific for each amino-acid might possess a unique content of methylated bases. They have shown that purified seryl-transfer RNA contains only a dimethylguanine in addition to the four non-methylated bases.

In higher proportion than the methylated purines, the sRNA fraction contains the nucleotide, pseudouridylic acid, in which the uracil is attached to the ribose at the 5 ring position, that is through a C-C linkage. It represents 2-4% of the total nucleotide content.

The other significant feature of sRNA base composition is the equivalence of its purine and pyrimidine content (e.g. 56 and 57) and this observation supports other evidence that the molecules of sRNA possess a hydrogen bonded secondary structure, similar to that of double-stranded DNA. First indications of such an association were obtained from changes in the optical density and optical rotation which accompany changes in temperature and salt concentration of solutions of sRNA (48,59).

From the magnitude of these hyperchromic transitions, it was estimated that 40-60% of the bases are involved in hydrogen bonded interactions, the rest were assumed to be contained in non-hydrogen bonded loops (60). However, more recent X-ray diffraction studies on crystalline samples of the whole sRNA population of yeast have led to the proposal of a more detailed model (61) in which each molecule is in the form of a double helix. The helix is most likely formed by the molecule folding back on itself, and one important consequence of this is that in the region of the fold there must be at least three nucleotides whose bases are not involved in intramolecular hydrogen bonding. It has been proposed (61) that these might be the bases which interact with the template RNA at the ribosome during protein synthesis(62).

The Ribosomes and ribosomal RNA.

The occurrence of the ribosomes and the methods used in isolating them have been described in a previous section. A point often emphasised is the similarity of the ribosomes derived from a variety of sources. Variations in particle weight between the ribosomes of different sources and from the same source under different conditions of growth,

have been demonstrated (63). However the composition of the protein component derived from a variety of ribosomes and from plant viruses show similarities (64). Comparison of the ribosomal nucleic acid base composition shows even more striking similarities (63). Base analysis also shows that the content of the methylated purines and pseudouridine is negligibly low in ribosomal RNA (65). A purine to pyrimidine ratio close to unity is found for this fraction and, as with sRNA, evidence for secondary structure is obtained from changes in optical density and optical rotation on heating (66). Both ribosomes and the RNA derived from ribosomes demonstrate this phenomenon.

Examination of the sedimentation properties of ribosomes has shown that the particle weight distribution is not polydisperse, but lies in discrete fractions. (25,67). These fractions are referred to by the nearest integer to their sedimentation coefficient (e.g., 30S, 50S and 70S ribosomes). The larger ribosomes owe their integrity to the presence in the surrounding fluid of magnesium (25,68). The polyamines putrescine and spermidine also impart stability to the ribosomes of Escherichia coli (27). A decrease in the magnesium ion concentration leads to an increase in the number of particles in the low weight

fractions. This process is reversed by increasing the magnesium concentration, which suggests that the larger are aggregates of the smaller ribosomes. The way in which these additives stabilise the ribosomes is not known, but Zamecnik (62) has suggested that magnesium ion might be responsible for the attachment of messenger RNA to the ribosome.

Phenol and certain detergents, denature the protein component and free the ribosomal RNA (69). RNA isolated in this way from 70S ribosomes (28S RNA) (70) demonstrates a reversible dissociation dependent upon the magnesium concentration and it has been suggested that this might account for the dissociation of 70S ribosomes to 50S and 30S ribosomes in the absence of magnesium.

Present knowledge concerning the enzymic synthesis of ribonucleic acids and other aspects of their metabolism.

The discovery of RNA polymerase and the elucidation of the template mechanism by which it is controlled, represents a major advance in our knowledge of the ways in which the cell synthesises its diverse population of ribonucleic acids. The enzyme, first

reported by Weiss (71), incorporates ribonucleoside-5'-triphosphates into polynucleotides by a reaction which depends on the presence of all four triphosphates and DNA. Its presence in many bacterial (72,73,74), animal (71,75,76), and plant (77) cells has been demonstrated. DNA derived from a variety of sources fulfils the primer requirement. The reactions in the presence of the synthetic deoxyribopolynucleotides poly dT and poly dAT are of particular interest. In the first case only ATP is incorporated and the product is poly A (78). If poly dAT is the primer the presence of both ATP and UTP is necessary for formation of a ribopolynucleotide whose base sequence, an alternation of uracil and adenine, exactly mimics the alternating sequence of adenine and thymine in the primer (79). These and other less definitive results (73,80) provide evidence that the base sequence of the ribopolynucleotide product is complementary to that of the DNA template.

Both double-stranded, native DNA (81) and single-stranded (poly dT (78), $\phi$-174 DNA (80)) deoxyribopolynucleotides are able to initiate the reaction. In this respect, RNA polymerase differs from DNA

polymerase which requires a single-stranded DNA for priming (83). In the absence of any primer molecule, the RNA polymerase of Micrococcus lysodeikticus is able to catalyse a slow incorporation of ATP and UTP only when these are present together. Separate chains of poly A and poly U are formed (84).

The relationship of the ribopolynucleotide product to the deoxyribopolynucleotide primer suggests that RNA polymerase might be responsible for the synthesis of those RNA molecules for which there exists, in the nucleus, a complementary stretch of DNA. This is one of the characteristics which have been used to define the messenger RNA fraction and Spiegelman has recently reported (85) that ribosomal RNA of E. Coli bears a base sequence complementary to a small fraction of its homologous DNA.

The complementarity of the template and product also suggests that the reaction depends upon the formation of hydrogen-bonding between the bases of the ribonucleoside triphosphates and the DNA template. This hypothesis is supported by the observations of Kahan et al. (82) who

studied the incorporation of synthetic nucleotide analogues by RNA polymerase. It was found that only nucleoside triphosphates whose bases were able to form hydrogen bonds to the complementary base were incorporated into the ribopolynucleotide.

Hydrogen-bonded interactions between ribopolynucleotides similar to those which occur between DNA-DNA and RNA-DNA complementary strands, are known to occur (87) and one might expect that enzymes which replicate RNA using a RNA template are in existence. Weiss has shown (88) that the RNA polymerase of M. lysodeikticus is promoted by natural and synthetic ribopolynucleotides, though rather less effectively than by DNA. Enzymes of this type which have an absolute requirement for a RNA template have not yet been isolated but recent results suggest that such enzymes are induced when RNA viruses infect bacterial (89) or mammalian cells (90).

RNA dependent enzymes whose activity is promoted by a priming action which does not involve a template mechanism are of common occurrence. Frequently they differ from the polymerase system in that the primer molecule is incorporated into newly formed polynucleotide.

The many enzymes which belong to this general class differ widely in properties and in only one case can a well defined biological function be assigned. Thus, regeneration of the pCpCpA end sequence of amino-acid transfer RNA molecules is carried out by an enzyme which adds all three units in correct sequence from two molecules of CTP and one molecule of ATP. The enzyme shows stringent specificity for sRNA which has lost all or part of the terminal pCpCpA grouping (91); degradation of sRNA beyond this destroys its ability to act as acceptor. Other types of RNA (ribosomal, TMV) are inactive (92). It seems likely that a single enzyme catalyses the incorporation of all three nucleotide units and also the reverse reaction in which the triphosphates are formed by pyrophosphorolysis of the end group. The enzyme has been partially purified from mammalian (93) and bacterial cells (92,94).

Polynucleotide phosphorylase, which was the first enzyme capable of ribopolynucleotide synthesis, to be discovered (95) belongs to the class of enzymes whose action is promoted by a ribopolynucleotide primer.

Reaction involves the formation of 3'-5'-phosphodiester linkages from nucleoside-5'-diphosphates, with the elimination of inorganic orthophosphate:

$$nX\text{-}R\text{-}P\text{-}P \rightleftharpoons (X\text{-}R\text{-}P)_n + nPi$$

Enzymes of this type are found in a wide variety of bacteria (96) but evidence for their presence in animals and plants is scarce (96). Thorough studies of partially purified preparations from various bacterial sources have divulged differences in the properties of some of them. In general, the enzymes catalyse two other reactions in addition to polymerisation; viz. phosphorolysis of ribopolynucleotides, which is the reverse of polymerisation, and exchange of the terminal phosphate group with inorganic orthophosphate:

$$XR\text{-}P\text{-}P + Pi^{32} \rightleftharpoons XR\text{-}P\text{-}P^{32} + Pi$$

In most cases the exchange, phosphorolysis and polymerisation appear to be carried out by the same enzyme, but in Clostridium perfringens there is evidence that these reactions are carried out by different enzymes (97). The enzyme of Azoterbacter agilis was the first to be discovered and it has now been purified 500-fold (98). Early studies (99) showed that poly-

nucleotide phosphorylase of A. agilis is capable of incorporating single nucleoside pyrophosphates or any mixture of two or more of them into polymer, the base composition of which depends upon the composition of the mixture. The extent of purification is such that it is unlikely that more than one enzyme is involved. Homopolymers which are formed from single pyrophosphates (e.g. poly A from ADP, poly U from UDP) have proved useful as model compounds in studies of polynucleotide interactions (87) and of many enzyme systems. Magnesium is required for reaction and under certain conditions of magnesium /diphosphate concentration, there is a lag phase before polymerisation commences. Lag phases also occur with enzymes prepared from M. Lysodeikticus (100) and E. Coli (Section II of this thesis). It is found that the lag phase is overcome by the addition of oligonucleotides or long chain polynucleotides to the reaction mixture. If the primer molecule contains a free 3'-hydroxyl group it is incorporated into the newly formed polynucleotide. Primer molecules with 3'-phosphate end groups are not incorporated although they do overcome the lag phase, at least in the case of the polymerisation of UDP or ADP by the Azoterbacter enzyme (101). The priming

action of long chain polynucleotides shows some specificity with regard to the nature of the base. Thus, for instance, poly A synthesis is stimulated by poly A, poly C, poly AU and RNA or poly AGUC but inhibited by poly U. Conversely, polyU synthesis is stimulated by poly U and inhibited by poly A. Poly C stimulates the formation of all types of polyribonucleotides including poly G, which is otherwise very slowly incorporated into polymers of low molecular weight. Primer effects are discussed more fully in a review by Grunberg - Manago (102). As yet these effects are not fully understood and no pattern has been found which gives an indication as to how polynucleotide phosphorylase might produce RNAs of well defined composition and base sequence. E. Coli and M. Lysodeikticus, like A. agilis, each appear to contain a single enzyme which catalyses the three reactions of polynucleotide phosphorylase and which operate on all four nucleoside diphosphates. Experiments carried out with Cl. perfringens (97) have already been mentioned; in the present connection, the same authors have shown that this organism may contain separate enzymes for the polymerisation of each nucleoside-5'- pyrophosphate. It appears fairly conclusive that for ADP and GDP

polymerisation there are two distinct enzymes and that of two ADP polymerising enzymes, one is unable to catalyse the phosphorolysis of poly A. Furthermore, this same enzyme is stimulated not by polynucleotides but by basic polypeptides such as polylysine.

The apparent lack of mechanisms which control the base composition and sequence of the polymers synthesised by polynucleotide phosphorylases, has raised doubts as to whether their role in cellular metabolism is the synthesis of polynucleotides. The possibility that they are involved in the degradation of certain types of RNA is supported by the fact that in extracts of E. Coli messenger RNA is degraded to 5'-nucleotides (103). With few exceptions (see later), ribonucleases degrade RNA to 3'-nucleotides. In considering the role of polynucleotide phosphorylase, the possibility that non-specific polynucleotides such as those synthesised by this enzyme, might provide a means of storing mononucleotides in certain cells, appears to have been overlooked. Polynucleotide phosphorylase could function not only in synthesising such a storage polynucleotides but in providing from them the nucleoside diphosphates which

are readily converted into a form available for specific RNA synthesis.

A number of studies of the utilisation of radioactively labelled nucleoside triphosphates by crude or partially purified cell free systems has revealed the occurrence of several enzymes which incorporate these materials into ribopolynucleotides. In general, proof of incorporation into polynucleotides rests on the demonstration that radioactivity appears in acid insoluble material from which it is released by the action of alkali or ribonuclease. A refinement of this technique enables a distinction between "terminal" and "non-terminal" incorporation to be drawn. Nucleotides in the terminal position appear as nucleosides on alkaline hydrolysis, while non-terminal nucleotides produce nucleoside monophosphates. It was by this approach that Weiss demonstrated the presence of RNA polymerase in rat liver (71).

Other enzymes which catalyse both terminal and non-terminal incorporation from nucleoside-5'-triphosphates have been found in a variety of organisms. Few of these are well characterised, but clear distinctions in their

properties have been shown. The examples quoted in the following paragraphs illustrate these distinctions.

Enzymes which are specific for the incorporation of AMP from ATP have been found in calf thymus nuclei (104) and E. Coli ribosomes (105). The purified enzyme is able to synthesise long chain polyadenylate and appears to require the presence of a RNA which contains a sequence of adjacent adenylate units (a synthetic polyadenylate will serve The discovery of small amounts of polyriboadenylate among the RNAs of calf thymus (106) suggests that the enzyme might be responsible for the synthesis of such polymers, in vivo.

In spinach leaves (107) and in E. Coli infected by λ-virus (108) are found enzymes which require the presence of all four nucleoside triphosphates. The spinach leaf enzyme is active only if a RNA primer molecule is incubated with the protein before addition of the triphosphates. This enzyme has been named RNA synthetase.

Enzymes which are more active in incorporating

UTP than the other triphosphates are found in pigeon liver microsomes (109) and rat liver ribosomes (110). The primer requirement of these enzymes is apparently non-specific, ribosomal RNA and even ribonucleotidyl DNA will serve to initiate the reaction.

Ribonucleotidyl DNA is synthesised by an enzyme from calf thymus which is able to effect the incorporation of a limited number of ribonucleotides or deoxyribonucleotides into terminal positions of DNA molecules (111). Single triphosphates or mixtures are used by the enzyme. A similar enzyme from E. Coli (112) is stimulated by the addition of DNA polymerase and, for the incorporation of $P^{32}$-CTP, requires the presence of dATP,dGTP, and dUTP. The biological significance of this interesting reaction remains obscure.

The incorporation of UMP into RNA from $P^{32}$-UTP by extracts of Ascites carcinoma has been studied by Burdon and Smellie (113). Using crude extracts, the nucleotide appears in both terminal and non-terminal positions but the enzymes responsible for these types of incorporation can be separated by ammonium sulphate fractionation.

An enzyme which has been purified from calf thymus adds only cytidyl residues to terminal positions of thymus RNA (114).

Although the enzymes listed above are not fully characterised and cannot yet be fitted into a metabolic scheme, their existence implies that synthesis of RNA in living cells is not carried out by DNA-dependent RNA polymerases alone. At present, however, these enzymes represent the only known mechanism available to the cell for the synthesis of RNA whose base sequence is well defined over the whole length of the chain.

A few enzymic transformations of intact polynucleotides have been reported and reactions of this type may prove to be important in RNA metabolism. Best defined among these is the methylation of the bases of soluble RNA, by an enzymic transfer from S-adenosyl-methionine (115). Synthetic RNA and DNA are also methylated by this system (116). It has been shown (115) that sRNA of a methionine-less mutant of E. Coli is deficient in methylated bases. This sRNA is methylated by the methionine system and it seems likely that this is the mechanism by which these bases arise in normal cells.

Colowick has reported (117) the presence of an enzyme in rat muscle extracts which catalyses a

reversible phosphorolysis of guanine bases from intact RNA. The residual RNA, precipitated as the salmine salt, contains acid labile phosphate (presumably ribose-1-phosphate) equivalent in amount to the guanine which is removed (118). No evidence for the presence of glycosyl phosphate groups in RNA has been reported, though evidence for their occurrence, to a small extent, in DNA has been presented (119,120). In the light of this it seems that the enzyme might be an artifact. However, such an enzyme would provide a basis for the results obtained by Brown et al. (121) who administered nucleotides labelled in the base, sugar and phosphate groups with $N^{15}$, $C^{14}$, and $P^{32}$ respectively, to whole rats. It was found that 80% of the guanine residues in the isolated RNA had become attached to a new ribose moeity. This transfer was much more pronounced with guanine than with any other base and to account for it the authors proposed a "transpurination" mechanism and considered the possibility that this might occur by transfer of the base to a pre-existing RNA backbone (122). However a transfer at the nucleotide level could also explain the results.

Experiments with labelled compounds carried out

with whole animals are often extremely difficult to interpret owing to the complexity of the system and more recently the problem has been partially simplified by studying the utilisation of radioactive RNA precursors by unicellular organisms and cells cultured in vitro. Attempts have been made using this technique to determine the intracellular site of RNA synthesis, the time course of the process, and the fate of the product. Some progress towards these objectives has been made, in spite of the limitations of the method, which most frequently involves the acid fixation of cells grown in the presence of tritiated nucleosides. Subsequently the location of the radioactive "RNA" is determined by autoradiography.

Cells of many types including salivary glands of Chironomus tentans (123), ovary follicles of Drosophila (124) E. Coli (125), and starfish oocytes (126) have been studied and in each case it was found that incorporation is most rapid into the nucleus. Within the nucleus, the nucleolus reaches a higher specific activity than the chromatin bodies. The cytoplasm becomes labelled after a much longer lapse. In several reports, data on the kinetic relationship of nucleolar, chromatin and cytoplasmic labelling are claimed

to indicate metabolic relationship of the RNA fractions. To give but one example: autoradiographic studies of the liver and pancreas of mice which had been injected with $H^3$-cytidine led Amano and Leblond (127) to conclude that RNA synthesis occurs independently in the chromatin and nucleolus. Cytoplasmic RNA appeared to derive from the chromatin and the nucleolar RNA to be degraded in the nucleolus. Other workers have interpreted their own results in a quite different way and it is clear that the technique, as it is applied at the present time, cannot give a satisfactory answer to this very complex question. A comprehensive review of this type of work is given by Abrams (128) who draws attention to weaknesses in the technique which might lead to artificial results.

Determinations of RNA synthesis in enucleated cells and with cells whose nuclei have been wholly or partially damaged by irradiation indicate a close dependence of RNA synthesis on the presence of an intact nucleus. With enucleated portions of Human amnion cells, Goldstein et al (129) failed to demonstrate the incorporation of purines and purimidines into cytoplasmic RNA. However, incorporation of $P^{32}$ phosphate and $C^{14}$ adenine is continued by enucleated acetabularia (130) in a way

which suggests that turnover rather than net synthesis of RNA is responsible. By irradiating the nucleolus of HeLa cells with a microbeam of ultra-violet light Perry et al. (181) found that incorporation of tritiated cytidine into cytoplasmic RNA fell by 65% and into extranucleolar parts of the nucleus by 30%. It was concluded that synthesis of these portions of RNA are dependent on the nucleolus.

Attempts to present a comprehensive picture of RNA biosynthesis must await further information, in particular of the enzyme systems involved. Conclusive experimental evidence shows that the nucleus is closely involved in a high proportion of RNA synthesis and the DNA dependence of RNA polymerase implies that it must function in the nucleus. This mechanism is aligned with current theory regarding the transfer of genetic information, by the proposal that RNA polymerase is involved in the synthesis of the messenger RNA fraction. The synthesis of ribosomal RNA by this enzyme has also been suggested. The end group of sRNA is almost certainly formed by an enzyme which is known to be specific for that function, but the source of the main chain is obscure. The methylated bases probably arise by transfer of methyl groups to a

preformed chain. The widespread occurrence of polynucleotide phosphorylase and the many enzymes which utilise the nucleoside triphosphates, apparently without the operation of a mechanism for controlling the base sequence, suggest a function, as yet obscure, for enzymes of this type.

In view of the rapid turnover of RNA, in particular of messenger RNA, by the living cell, it is clear that degradation is an important process in the overall metabolism of RNA. Enzymes which degrade RNA are ubiquitous and vary widely in their properties. Most common are those which hydrolyse the 3'-5'-phosphodiester linkage and among these, those which produce the nucleoside-3'-monophosphates are most frequently encountered. Several distinct enzymes are included in this class. Ribonucleases of animal tissues are usually specific for phosphodiester linkages involving the 3'-hydroxyl group of a pyrimidine nucleoside, [e.g. pancreatic (132), spleen and liver ribonucleases (133,134)]. Takadiastase has been shown to contain two ribonucleases both of which are specific for linkages adjacent to purine nucleotides (135,136,137).

Enzymes which degrade RNA completely to nucleoside-3'-phosphate occur in plant leaves (138,139) and E. Coli ribosomes (140).

Degradation to oligonucleotides with 5'-monophosphateend-groups is achieved by an enzyme from A. agilis (141). Degradation ceases at the dinucleotide stage.

Under certain conditions, polynucleotide phosphorylase degrades RNA to the nucleoside-5'-diphosphates and this reaction appears to be affected by the secondary structure of the substrate (142). Thus synthetic polynucleotides and TMV which are single stranded are readily phosphorylised to near completion. sRNA and certain mixtures of synthetic polynucleotides which contain a good deal of H-bonded secondary structure are resistant to phosphorolysis.

It is clear that the nucleases must be concerned with the degradation of RNA, but no indications as to their part in the overall metabolism of the cell can be gained from present knowledge of their mode of action or cellular environment. It is worth noting that the 3'-phosphates, which are produced by most nucleases, cannot be

reutilised directly for the synthesis of RNA. According to the mechanisms so far discovered for RNA biosynthesis, conversion to a 5'-polyphosphate via the 5'-monophosphate would be a necessary prior step. Degradation to a 5'-phosphate or 5'-pyrophosphate, as is the case with polynucleotide phosphorylase, would lead to products which are more readily utilised for RNA synthesis.

Polyribosephosphate; its structure, occurrence and relation to RNA.

Polyribosephosphate (PRP) was first recognised and isolated as the type specific substance of Haemophilus influenzae type b (143). Zamenhof and his co-workers purifiedthe material and presented evidence (144) that it consists of two 3-5-linked ribosephosphate strands, each joined to the other via the reducing groups of the ribose moieties. Thus, a single strand would correspond to the backbone structure of RNA.

Acid hydrolysis of PRP produces equimolar quantities of ribose and ortho-phosphate. Alkaline hydrolysis gives rise to a carbohydrate phosphate which, on enzymic dephosphorylation, yields a non-reducing

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Acid hydrolysis of PRP produces equimolar quantities of ribose and ortho-phosphate. Alkaline hydrolysis gives rise to a carbohydrate phosphate which, on enzymic dephosphorylation, yields a non-reducing

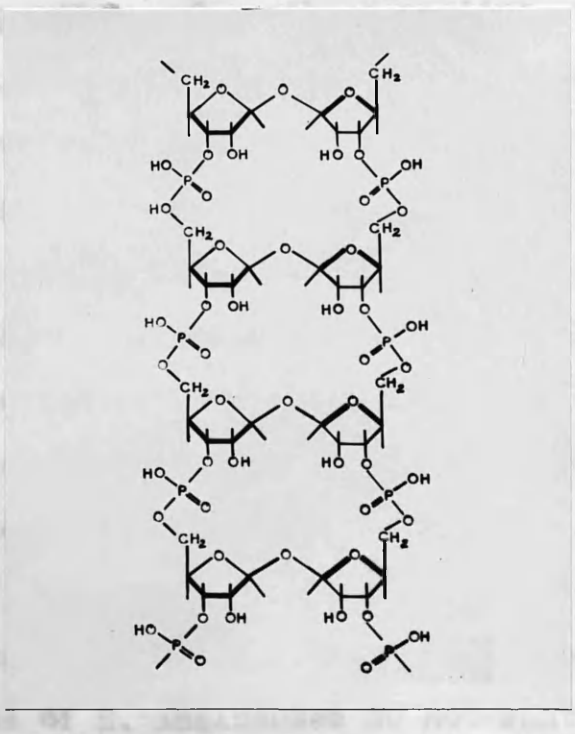
pentose derivative. This has been shown to be identical with synthetic β-1'-ribofuranosyl-β-1'-ribofuranoside (145). Evidence for the similarity of the phosphodiester linkage to that of RNA derives from electrometric titration, alkaline depolymerisation and the stability to periodate oxidation of the product of alkaline hydrolysis (144). The similarity is emphasised by the fact that PRP is depolymerised by pancreatic ribonuclease and ribonuclease A (145). The structure of polyribosephosphate is illustrated in Fig. 3.

In H. influenzae b, which is an encapsulated micro-organism, the PRP is apparently present in the capsules, for when suspensions of the bacterium are agitated, as in shake cultures, the PRP is released into the culture medium. A similar substance is found in the liver of the squid (146) and it has been pointed out (144, 145) that in other organisms it may have been overlooked by having been classified as RNA because of its orcinol-positive reaction.

The close structural relationship between PRP and RNA led Zamenhof et al. (144) to suggest that they might be related metabolically. The same group have

FIG. 3.

The structure of polyribosephosphate.



drawn attention to the fact that the similarity in energy between an O-glycosidic bond and a N-glycosidic bond is compatible with the possibility that one serves as a precursor of the other.

The occurrence of many enzymic reactions which involve the transfer of a nitrogenous base to the glycosidic position of a ribose residue supports this suggestion (147,148,149,150). In most of these reactions, the transfer involves an exchange of bases:

$$(\text{Ribose})\text{-}B_1 + B_2 \rightleftharpoons (\text{Ribose})\text{-}B_2 + B_1$$

or a reversible phosphorolysis:

$$\text{Ribose-1-phosphate} + B \rightleftharpoons \text{Ribose-}B + Pi$$

An enzyme of the latter type which catalyses a reversible phosphorolysis of guanine from intact RNA (117,118) has been referred to in a previous section.

In the present connection, it is noteworthy that autolysates of H. influenzae do not contain enzymes which are capable of degrading PRP (145); a fact which might be taken to indicate that PRP is not concerned in a degradative pathway in this organism. However, this result alone is slender evidence indeed for such a conjecture.

Scope of the present work.

In order to test the possibility of transferring bases to a preformed RNA backbone polymer, this material would be required in quantity. The work described in this thesis was directed towards producing such a polymer on a large scale.

In the following paragraphs, various alternative approaches to obtaining a RNA backbone polymer are discussed. The reasons for selecting the approaches tested in the present work and for discarding others are given. The experimental work which evolved from these concepts is briefly outlined. Much of what follows is contained in the main text but, at the risk of repetition, this brief abstract is presented to give a continuity which it is not possible to maintain in the more detailed description.

In H. influenzae b, the concentration of polyribosephosphate is very low; Zamenhof et al. (144) report a yield of approximately 700 micrograms from 90 agar plates. Thus, to produce even small amounts of the polymer large quantities of the bacterium are required and this precludes it as a source for the material on a large

scale. In view of this, alternative means of producing the polymer must be considered.

If it were possible to reproduce, in vitro, the system which the bacterium uses to synthesise the polymer this might provide a means of preparing it in quantity. Unfortunately, this mechanism is completely obscure.

The enzyme polynucleotide phosphorylase was first reported in 1955 (95) and at the time this work was initiated, its properties were fairly well defined. Of particular interest in the present context, was the finding that the enzyme shows a rather broad specificity towards the nucleoside-5'-pyrophosphates which it is able to incorporate into polyribonucleotides, and especially interesting was its ability to form homopolymers containing a single base. This lack of specificity with regard to substrate and product encouraged an attempt to synthesise a ribose phosphate polymer by the action of polynucleotide phosphorylase on ribose-5-pyrophosphate [the term ribose phosphate polymer is used throughout to distinguish the synthetic, single-stranded polymer from the natural, double-stranded polymer for which the name polyribophosphate

was coined by Zamenhof et al. (144)]. Requirements for this project were ribose-5-pyrophosphate and polynucleotide phosphorylase. The former was synthesised from ribose-5-phosphate, which was prepared by a new chemical synthesis from ribose. Polynucleotide phosphorylase was partially purified by a novel route from E. Coli. It will be shown later that this approach to the synthesis of a ribose phosphate polymer was not successful because of the failure of the enzyme to use R5PP as a substrate.

Attention was then turned to the possibility of removing the bases from a polyribonucleotide. At the time there had recently appeared a report of the preparation of a deoxyribose phosphate polymer (151) which was obtained from DNA by acid hydrolysis to remove the purine bases followed by treatment with anhydrous hydrazine to remove the pyrimidines. It was envisaged that by analogous means (though not necessarily using the same reactions) a ribose phosphate polymer might be prepared from a polyribonucleotide. For reasons discussed in the introduction to section III, the polyribonucleotide which is most likely to yield to this approach is polyuridylic acid and this was synthesised from UDP using

polynucleotide phosphorylase. The nucleoside pyrophosphate was synthesised in three steps from uridine using modifications of standard procedures.

In addition, for some model experiments, short chain polyuridylic acid was prepared by chemical synthesis.

The work outlined above is described in detail in Sections I and II . For convenience of presentation, the order of description departs from the order in which the work was carried out. All of the work connected with synthesis of ortho- and pyrophosphate esters (R5P, R5PP, U5P and UDP) is contained in Section I. Section II is concerned with the synthesis of polymers and related topics and in particular with the partial purification of polynucleotide phosphorylase.

Section III is a description of work which was directed towards removing uracil residues from intact polyuridylic acid. Preliminary tests of four methods for uracil removal on chemically synthesised polyuridylic acid appeared to show that none of these methods gives rise to

product with ribose moieties free in the 1-position. A reinvestigation of two methods with uridine-5'-phosphate showed that one of them is able to convert this compound wholly to ribose-5-phosphate.

Two methods were therefore applied again to chemically synthesised polyuridylic acid and the products analysed with greater care. It became apparent during the course of these experiments that a standard procedure for the determination of reducing power ( an alkaline copper method) gives invalid results with phosphate esters of ribose. Since this method had been used to determine the reducing power of polyuridylic acid, treated to remove the uracil, it was clear that this had provided false results in earlier experiments. To overcome this difficulty, a novel method for the quantitative determination of the reducing power of pentose compounds, which are sensitive to alkali, was developed. This technique, applied to products from polyuridylic acid after removing the bases, showed that one method produces a polymer whose ribose moieties are 80% uncombined in the reducing position.

The nature of this polymer and other new polymers produced by methods which are less successful in removing the uracil residues are discussed in a brief conclusory section.

In the Appendix, reaction mechanisms involved in the hydrolysis of N-glycosides and phosphate esters, which are relevant to the work in Section III, are discussed briefly.

## SECTION I

### Synthesis of orthophosphate and pyrophosphate esters.

The pyrophosphate esters of ribose and uridine, required for the projected polymer synthesis using polynucleotide phosphorylase, were obtained by chemical synthesis, via the corresponding orthophosphate. Natural sources which present a possible alternative for UDP, were not considered because of the lengthy procedure involved in isolating the ester (152).

Enzymic synthesis of UDP (153) using a kinase in in conjunction with ATP, is also a feasible route but possesses no advantage over chemical synthesis.

For similar reasons chemical synthesis was chosen as a route to the orthophosphate esters required for preparation of the pyrophosphates.

### Synthesis of orthophosphate esters: General.

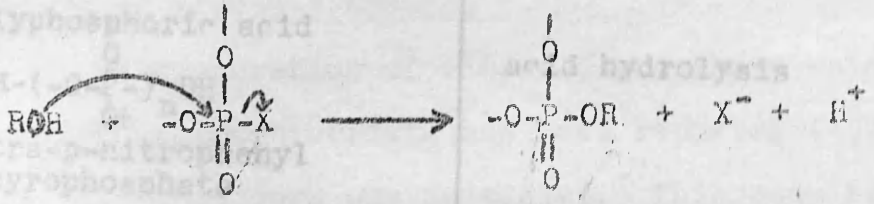
In recent years, a wide variety of methods for synthesising monophosphate esters, many of which are important in biological processes, have been developed (154, 155). Some of the general problems which are presented by these syntheses will be considered in the

following paragraph and in particular those which apply to the synthesis of nucleotides and sugar phosphates.

First, the nucleosides, nucleotides, and sugar phosphates are mostly labile compounds and comparatively mild conditions must be employed in any procedure in which they are involved. Secondly, as the nucleosides and sugars possess several functional groups at which ester formation is possible, it is frequently necessary to block all positions except those at which phosphorylation is desired. Fortunately, reagents are available which introduce blocking groups into the 2:3-positions of ribofuranose compounds [the isopropylidene (156) and benzylidene (157)] and the primary hydroxyl group at the 5-position is readily blocked in a specific manner [by the trityl group (156)]. All of these groups are easily removed under mild conditions. In certain cases protection is unnecessary because one position is much more highly reactive to ester formation than all others. This is the case for the 5-hydroxyl group in ribofuranose compounds.

Most phosphorylating agents are orthophosphates

which have been activated by anhydride formation either as phosphorochloridate or as pyrophosphate. An exception to this is β-cyanoethyl phosphate which is used in conjunction with the condensing agent, dicyclohexyl carbodiimide (DCC). Frequently the other hydroxyl functions of the orthophosphate are blocked to prevent condensation to pyro- and higher metaphosphates. Blocking these acidic hydroxyls also reduces the negative charge surrounding the phosphorus atom, and makes it more open to nucleophilic attack. The mechanism of the reaction is:

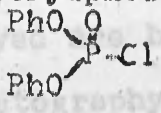
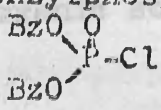
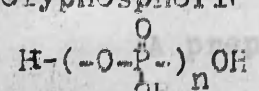
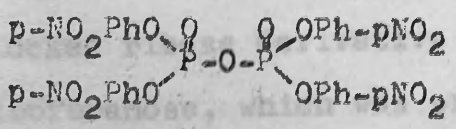
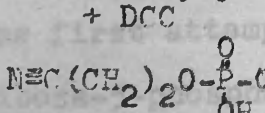


X = Cl or phosphate.

As with the groups used in blocking the hydroxyls of the carbohydrate, the choice of blocking groups for the phosphorylating agent is limited by the condition that the group must be readily removed under mild conditions. A list of the more common agents with their structures and the methods of removing the blocking groups is given in Table I.

Table 1.

Phosphorylating agents used in the synthesis of nucleoside- and sugar-phosphates.

| reagent: name and structure | method used to remove blocking groups | Ref. |
|---|---|---|
| diphenylphosphorochloridate  | hydrog. over Pt | (158) |
| dibenzylphosphorochloridate  | hydrog. over Pd | (160) |
| polyphosphoric acid  | acid hydrolysis | (162) |
| Tetra-p-nitrophenyl pyrophosphate  | alkaline hydrolysis | (161) |
| 2-cyanoethylphosphate + DCC  | alkaline hydrolysis | (163, 164) |

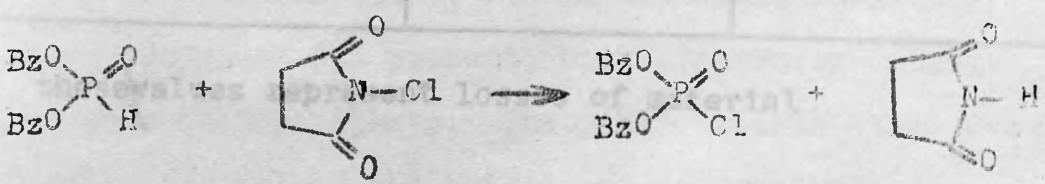
In addition to the difficulties involved in the phosphorylation reaction, there are problems associated with purification of the product. It is invariably contaminated with unreacted material and with inorganic orthophosphate which arises from the excess phosphorylating agent. The procedures most frequently employed are barium salt fractionation, ion exchange chromatography, adsorption chromatography on charcoal, and organic solvent fractionation.

Synthesis of ribose-5-phosphate.

A preparation of ribose-5-phosphate using dibenzylphosphorochloridate had been reported (157) at the time this work was initiated. This requires the blocked ribose derivative 1-O-methyl-2:3-O-isopropylidene-ribofuranose, which was prepared in good yield according to the method of Levene and Stiller (165) (Expt.1). The first attempt to repeat the original preparation of ribose-5-phosphate (Expt. 2) gave a very poor yield (10%) of the barium salt. The purity of the product was approximately 25% according to pentose analysis. An attempt was made in the second preparation (Expt. 3) to determine the stage at which this serious loss in

yield had occurred, by determining the total pentose (G.M. 1) present at each stage. The results of these analyses (Table 2) show that no serious loss of pentose occurs in any of the extraction procedures used in the preparation. Two explanations could account for the loss. Unreacted pentose might be carried through to the final step, if the initial reaction had not been successful. Alternatively, if the reaction did take place then the loss must have occurred in the barium salt fractionation. In this preparation as in the first, a sample of dibenzylphosphite which had not been purified by distillation was used to prepare the dibenzylphosphorochloridate.

In other experiments a purer sample [prepared according to the method of Friedman et al. (166)] of dibenzylphosphite was used and this was chlorinated by N-chlorosuccinimide:




This preparation avoids the hazards of distilling

Table 2.

Pentose and phosphate analysis at various stages during the synthesis of ribose-5-phosphate using dibenzyl phosphorochloridate.

| stage of process | total pentose (gms.) | inorganic phosphate(g.P) |
|---|---|---|
| 1) starting material | 6.3 | |
| 2) reaction mixture | 3.4 | |
| 3) solid after extraction with chloroform | 0.06* | |
| 4) aqueous washings of chloroform soln. | 1.5* | |
| 5) soln. before hydrogenolysis | 3.1 | |
| 6) after hydrogenolysis | 3.2<br>3.2 | 0.6 |
| 7) precipitate with barium hydroxide | 0.1* | 0.5 |
| 8) soln. before addn. of ethanol | 3.3 | 0.12 |
| 9) supernatant ethanol. | 3.2* | 0.03 |

* these values represent losses of material

dibenzylphosphite.

Two preparations were carried out using dibenzylphosphorochloridate prepared in this way and minor modifications in the isolation procedure improved the yield ofR5P from 8% (Expt.4) to 30% (Expt.5). Calculation of the yield at various stages in these preparations strengthened the view that the loss occurred in the final barium salt fractionation. In the last preparation, for example, the P,P-dibenzyl ribose-5-phosphate ester was isolated before removal of the benzyl groups and at this stage the apparent yield was 105%. Further purification of the compound by treatment with ion exchange resins reduced the weight by 25% (probably owing to the removal of dibenzylphosphate). After hydrogenolysis, the purity of the product was tested by paper electrophoresis (G.M. 6) [spots detected by fluorescence with quinine sulphate (167)] and it was found that only one major component was present, contaminated by a trace of inorganic orthophosphate. The yield at this stage was 73%. After removal of the methyl and isoproplyidene groups by hydrolysis with sulphuric acid, the barium salts were isolated by a modified procedure. In the

original preparation the sulphuric acid was neutralised by adding a mixture of solid barium carbonate and saturated barium hydroxide solution. The possibility that the phosphate ester might become adsorbed to the barium carbonate was avoided, in Expt. 5, by neutralising with potassium hydroxide and adding barium acetate to precipitate sulphate and inorganic orthophosphate. Barium ribose-5-phosphate was precipitated as before by the addition of ethanol. This modified procedure also avoids the dilution caused by adding large volumes of barium hydroxide solution, and the yield of barium salt was greatly improved (41%). A small amount of barium R5P was recovered from the precipitate of barium sulphate by treating it with dilute acid; these washings were neutralised and the Ba R5P recovered as before. This treatment increased the overall yield to 59.3%. Analysis of the two Ba R5P crops showed that the second was purer than the first (70% vs. 53%) according to pentose content. Taking this factor into account the overall yield of Ba R5P is 30%.

In view of the lengthy procedure involved in preparing the dibenzylphosphorochloridate

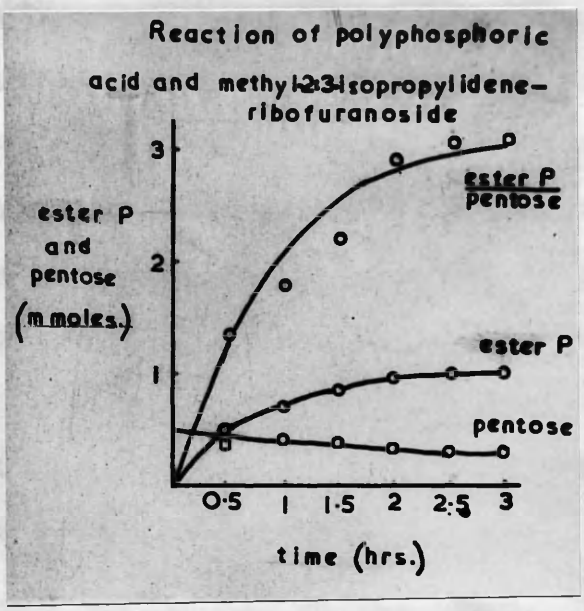
reagent and the moderate yields of R5P of low purity which were obtained by its application, it was decided to investigate some of the newer methods of phosphorylation which had appeared at this time. Several of these are listed in Table I. It was desirable that the reagent should be simple in preparation and use and that the yields be sufficient to provide enough R5P for attempts to prepare the pyrophosphate.

The reagent which fulfils the first condition most adequately is polyphosphoric acid. This reagent is prepared by dissolving phosphorus pentoxide in 85% phosphoric acid (G.M. 8) and the reaction is carried out by dissolving the substrate in this solution. In spite of the apparent severity of the reagent (particularly as a dehydrating agent) it has been used to prepare many phosphate esters including the labile pyridoxal phosphate (168) and the more stable pyrimidine nucleoside-5'-phosphates (169). It has also been used in the preparation of G6P (170). This reagent was tested in analytical scale experiments with 1-O-methyl-2:3-O-isopropylideneribofuranose and later with unprotected ribose. Its value in synthesising large quantities of R5P from unblocked ribose has been proved.

The small-scale experiments were carried out using the reaction conditions described by Hall and Khorana (162) to determine the optimum reaction time for the formation of R5P. The first reaction was carried out using 1-O-methyl-2:3-O-isopropylideneribofuranose which was mixed with an excess of polyphosphoric acid. The mixture was held at 60°C and aliquots, which were withdrawn at intervals, were diluted to stop the reaction. To hydrolyse the polyphosphates the aqueous solutions were boiled and inorganic orthophosphate removed by precipitation as the lithium salt. The supernatant solution was analysed for pentose and for ester phosphate (G.M. 1 and 2) in order to follow the course of ester formation. It was found that the precipitation procedure left a considerable amount of orthophosphate in solution and this interferes with the determination of ester phosphate. In the second experiment (Expt. 6), therefore, the inorganic phosphate was precipitated by neutralising with baryta, as described by Michelson (170) and the solutions treated in this way were made up to standard volumes. Aliquots from these diluted solutions were analysed for ester phosphate and for pentose. The results are shown in Table III where molar values of each component are given at various reaction

Table 3 and Fig.4.

Phosphorylation of 1-O-methyl-2:3-O-isopropylidene -ribofuranose by polyphosphoric acid.




| Reaction time(hrs.) | Pentose (mmoles) | Ester Phosphate (m.moles) | Molar ratio Phosph./Pentose |
|---|---|---|---|
| 0.5 | 0.37 | 0.5 | 1.35 |
| 1.0 | 0.4 | 0.71 | 1.8 |
| 1.5 | 0.37 | 0.81 | 2.18 |
| 2.0 | 0.33 | 0.97 | 2.94 |
| 2.5 | 0.30 | 1.0 | 3.33 |
| 3.0 | 0.30 | 1.0 | 3.33 |

times. In addition, the molar ratio of ester phosphate to pentose is shown. All these factors are plotted against reaction time in Fig. 4. It can be seen that the ester phosphate rises to a value higher than is expected for the formation of a monophosphate ester. However, the estimated pentose is less than half what is expected from the amount of 1-O-methyl-2:3-O-isopropylideneribofuranose used in the preparation. Thus it appears that the cause of disparity between the molar estimates of pentose and ester phosphate is interference in the colorimetric estimation by some unknown factor. Evidence will be presented that the product is, in fact, a monophosphate ester, identical with ribose-5-phosphate.

Because of the uncertainty which arises from the colorimetric estimations, paper chromatographic analysis (G.M. 5) was used in subsequent experiments to follow the course of the reaction. This method possesses the advantage that the fate of the ribose and the appearance of R5P can be followed together. Two parallel experiments (Expt. 7) were carried out in which 1-O-methyl-2:3-O-isopropylideneribofuranose and unblocked ribose were treated identically. The reaction conditions and working up procedure were identical with the experiment which

was followed colorimetrically (Expt. 6). It was evident from visual examination of the reaction mixture that the free ribose was charred by the reagent more rapidly than the blocked ribose. In addition, the blocked derivative was more soluble in the reagent. Aliquots of the reaction mixture were treated as previously, but after barium salt precipitation the supernatant solution was applied to Whatman No. 1 chromatography paper. The papers were developed in Solvent 1 and reducing sugars were detected by the alkali silver nitrate reagent (G.M. 7). Each aliquot from either experiment, showed only two spots on the chromatograms; one corresponding to ribose ($R_f$ =0.59) and the other to R5P ($R_f$ = 0.26). For both substrates, the ribose spot decreased as the reaction time increased , and the R5P spot increased in intensity until it was approximately equal to the ribose spot for the same reaction time. Thereafter both the ribose and R5P spots decreased in intensity. Thus, the optimum yield attainable by this method appears to be approximately 50%. For blocked ribose this was reached after 2-2½ hours and for free ribose after 1½-2 hours.

There was no apparent advantage in using the

the blocked derivative. The preferential formation of the 5-phosphate and the apparent non-formation of diphosphates when unblocked ribose is phosphorylated in this way may be accounted for by three factors. First, the 5-hydroxyl is the only primary hydroxyl group in the molecule and such hydroxyl groups are known to be more amenable to phosphorylation than secondary hydroxyls. Secondly, the 2- and3- phosphate esters, if formed, would be more labile to acid hydrolysis in the working up procedure, than the 5-phosphate. Alternatively, diphosphates might not appear in the chromatograms, because their barium salts are insoluble under the conditions used to precipitate the inorganic phosphate.

In a preparative scale experiment the only difficulty which arose was that of handling the large quantities of solution which are obtained when the inorganic orthophosphate is precipitated with barium hydroxide solution. Neutralisation with KOH and precipitation with $Ba(OAc)_2$ which led to an improved yield from dibenzylphosphorochloridate was not used in this case because the large excess of phosphoric acid present would have resulted in the formation of a large amount of

potassium phosphate, which may have led to difficulties. It was necessary to reduce the solution, obtained after precipitation of inorganic phosphate, to a much smaller volume before precipitating the BaR5P with ethanol. The yield of the barium salt was 10% of theory and according to pentose analysis (G.M. 1) the product was 80% pure. Paper chromatography in a variety of solvents including two-dimensional development in solvent 2 and3 , electrophoresis in several buffers including borate showed a single spot which corresponded with authentic R5P.

The product from this preparation was light brown in colour owing to the presence of charred material. In a later preparation (Expt. 9) this was removed by the addition of activated charcoal before ethanol precipitation of BaR5P. The yield in this experiment was 6%. It is not known whether the loss was caused by the decolorisation step or some other factor. The product, in this case, was white.

The yields in all the R5P preparations has been lower than expected from the initial extent of reaction and it is probable that the major part of this loss occurs in the final precipitation of the barium salt by ethanol, since this was the only step which was omitted in

the analytical experiment, which gave an apparent yield of 50%. Smaller losses may be caused by occlusion or adsorption of R5P by the precipitate of barium phosphate or sulphate. No better rapid method for the separation of R5P from inorganic phosphate is known. The modification used in Expt. 5 is recommended for the barium salt fractionation in the absence of a large excess of inorganic phosphate. Polyphosphoric acid is the reagent of choice for the preparation of R5P because of the simplicity of its use. The low yield is almost certainly caused by losses in the isolation procedure.

Preparation of uridine.

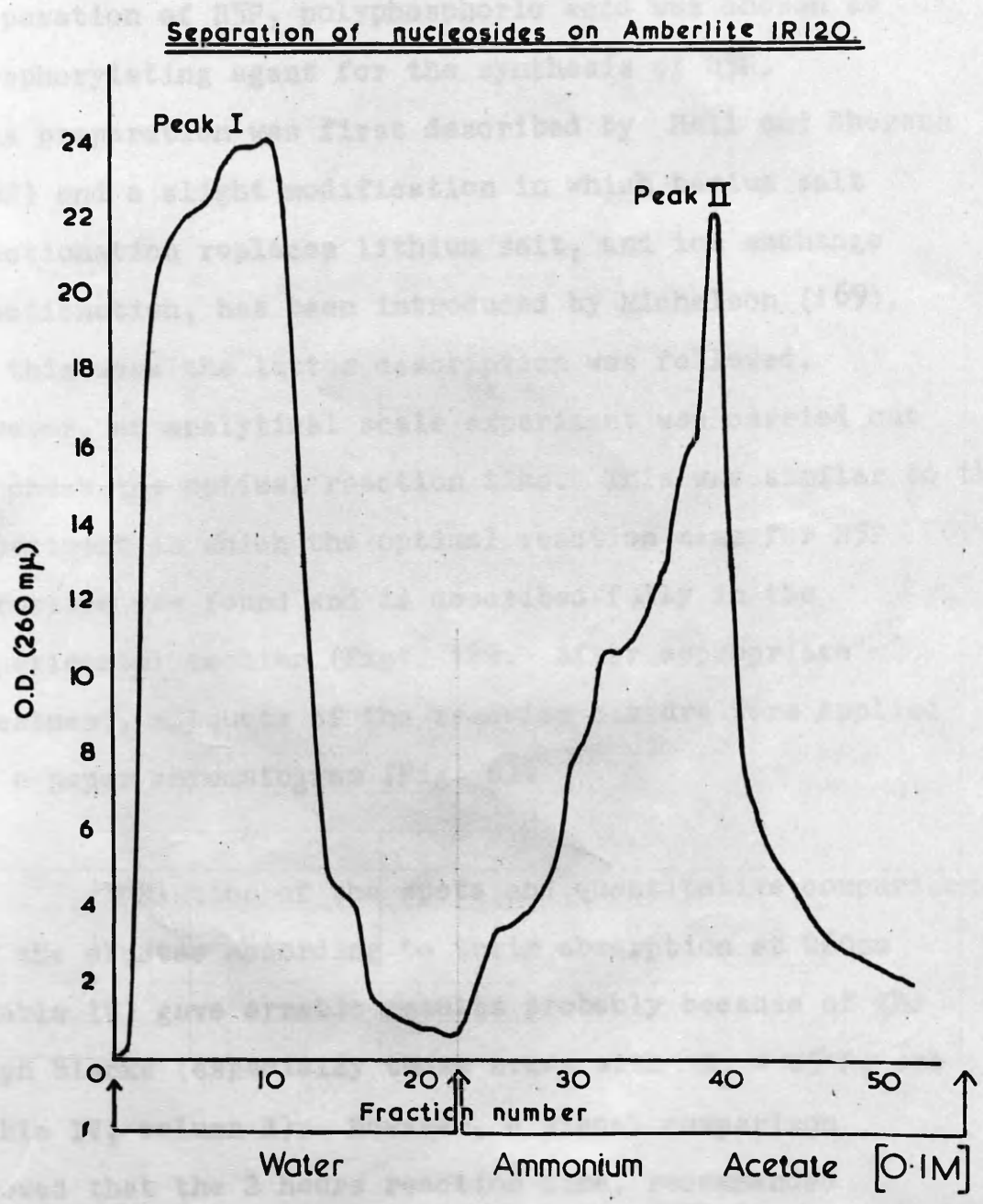
Uridine was required in large quantities for the synthesis of UDP. Several methods for the preparation of nucleosides from ribonucleic acid have been reported. Hydrolysis in aqueous pyridine introduced by Bredereck et al. (172) was used here. Commercial yeast nucleic acid (L. Light and Co. Ltd.) was hydrolised in refluxing 50% aqueous pyridine for 100 hours (Expt. 10). Examination of the hydrolysate by paper chromatography (G.M. 5, solvent 5) showed the presence of uridine, adenosine, guanosine, adenine and a trace of cytidine. Guanosine, which is

only slightly soluble in water, was easily removed by crystallising from this solvent, but it was necessary to employ an ion exchange resin (G.M. 9) to separate the other components from uridine. An analytical scale separation on Amberlite IR 120 is shown in Fig. 5. Peak I, which was not retained by the resin, contained only uridine as U.V. absorbing material and peak II, eluted with ammonium acetate (0.1M, pH 9.0), contains adenine and adenosine. The bulk of the hydrolysate was separated on a large column of Zeokarb 215 ($H^+$). Again, the uridine appeared in the first fractions. Adenine and adenosine were eluted with 0.1N ammonia. Evaporation of the fractions containing uridine produced a light yellow solid (16% of the weight of nucleic acid). Crystallisation from 95% ethanol yielded 30% of this in crystalline form. The rest was obtained as a yellow amorphous solid by evaporating the mother liquors.

Preparation of 2':3'-O-isopropylideneuridine.

Uridine obtained from yeast RNA as above was converted to its 2':3'-O-isopropylidene derivative by the method of Levene and Stiller,(173) in good yield and used without further purification (Expt. 11).

FIG. 5


Separation of nucleosides on Amberlite IR120.
Peak I
Peak II
O.D. (260 mμ)
24
22
20
18
16
14
12
10
8
6
4
2
0
10
20
30
40
50
Fraction number
Water
Ammonium
Acetate
[0·1M]

Synthesis of Uridine-5'-phosphate.

In the light of earlier experience with the preparation of R5P, polyphosphoric acid was chosen as phosphorylating agent for the synthesis of U5P. This preparation was first described by Hall and Khorana (162) and a slight modification in which barium salt fractionation replaces lithium salt, and ion exchange fractionation, has been introduced by Michelson (169). In this work the latter description was followed. However, an analytical scale experiment was carried out to check the optimal reaction time. This was similar to the experiment in which the optimal reaction time for R5P formation was found and is described fully in the experimental section (Expt. 12). After appropriate treatment, aliquots of the reaction mixture were applied to a paper chromatogram (Fig. 6).

Elution of the spots and quantitative comparisons of the eluates according to their absorption at 260mu (Table IV) gave erratic results probably because of the high blanks (especially those areas with $R_f$ = U5'P; see Table IV, column 2). However, a visual comparison showed that the 2 hours reaction time, recommended

Fig 6

Reaction of polyphosphoric acid and isopropylidene uridine

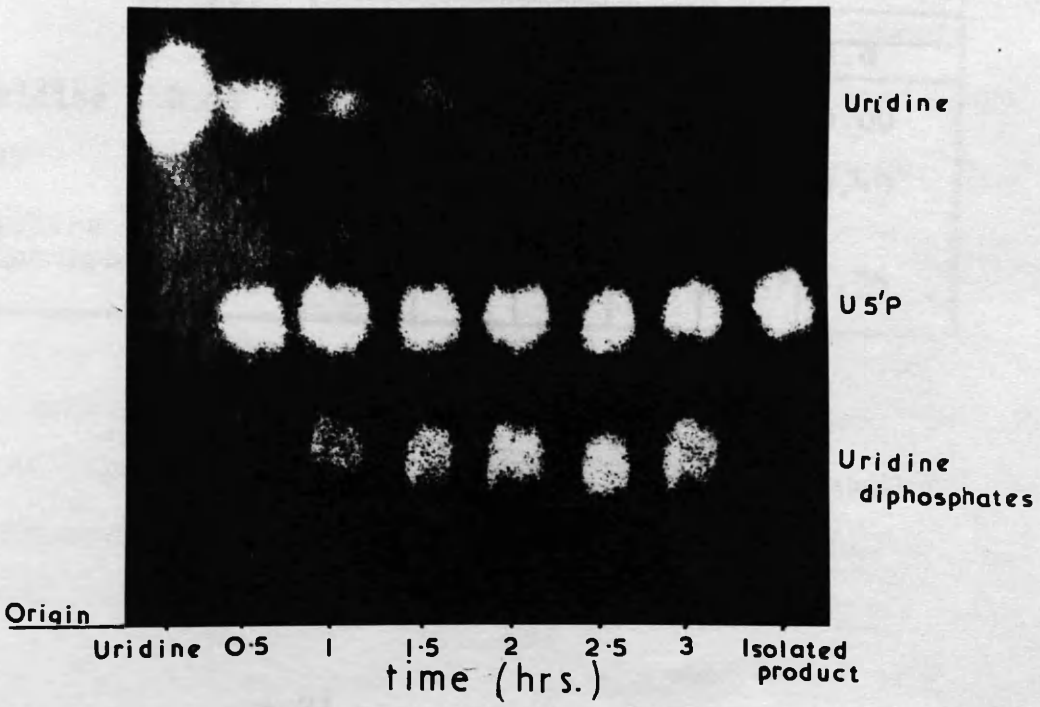


Table 4.

Optical density at 260 mμ of spots eluted from chromatogram shown in Fig.6

| Compound | Optical density at 260 mμ | | | | | | |
|---|---|---|---|---|---|---|---|
| | blanks Av.of 3) | Reaction time (hrs.) | | | | | |
| | | 0.5 | 1.0 | 1.5 | 2.0 | 2.5 | 3.0 |
| Uridine | 0.05 | 0.19 | 0.22 | 0.05 | 0.05 | 0.02 | 0.00 |
| U5'P | 0.19 | 0.31 | 0.51 | 0.44 | 0.45 | 0.39 | 0.40 |
| Uridine diphosph. | 0.07 | 0.06 | 0.17 | 0.20 | 0.28 | 0.27 | 0.36 |

both by Michelson and by Hall and Khorana is suitable.

A small-scale experiment (Expt. 13) in which the barium U5'P was isolated gave a yield of 74% of a material which was virtually free from uridine and uridine-2'(3'):5'-diphosphate as judged by paper chromatography (Fig. 6).

Further purification, by organic solvent fractionation (Expt. 13) showed that this incurs high losses of U5'P and was omitted in the subsequent preparations.

A large scale preparation (Expt. 14) gave a much lower yield (32%) of Ba U5'P. The reason for the loss in yield is not clear. One possibility is that the isopropylidene uridine used was not as pure as that used in the smaller scale experiment.

Synthesis of pyrophosphate esters: General.

The pyrophosphate bond is a particularly labile one and this must be taken into consideration in the preparation of pyrophosphate esters. Fortunately, the phosphate hydroxyl functions are acidic and

therefore more active as nucleophiles than alcoholic hydroxyl groups. This permits the use of milder reagents and reaction conditions in the phosphorylation of phosphate groups than are necessary for the formation of orthophosphate esters from alcoholic hydroxyl groups.

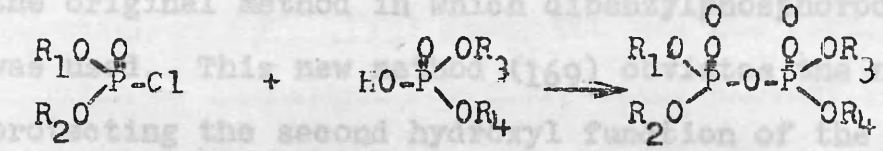
Some of the reagents developed primarily for the synthesis of orthophosphate esters have been adapted to formation of pyrophosphates. Dibenzyl phosphorochloridate was used in the first successful synthesis of a nucleoside -5'-pyrophosphate (174). In this early synthesis the substrate was P-monobenzyladenosine-5'-phosphate and the $P^1$-adenosine-5'$P^1$monobenzyl-$P^2P^2$-dibenzyl pyrophosphate ($R_4$=adenosine, $R_1$=$R_2$=$R_3$= benzyl in I, Fig.7) was converted to ADP by catalytic hydrogenation. The low yield in this synthesis and the difficulties involved in obtaining the protected nucleotide derivatives do not make it an attractive preparative method. A method which was developed by Khorana and his collaborators made use of the condensing agent dicyclohexylcarbodiimide (DCC). In its presence orthophosphate esters condense to form pyro- and higher polyphosphates. Nucleoside 5'-mono-

phosphates in the presence of excess inorganic orthosphosphate and DCC are converted mainly to the 5'-triphosphate. Some pyrophosphate and tetra-phosphate are also formed (175). This method is rarely chosen for the synthesis of pyrophosphates because of the low yields and the complexity of the products. However, the method was used in the only reported preparation of ribose-5-pyrophosphate (176). DCC is useful in many other condensation reactions and particularly relevant to this discussion is the formation of the phosphoramidates. These compounds are formed by the reaction of ammonia or an amine (e.g. morpholine) with a monophosphate ester in the presence of DCC. The phosphoramidates (or morpholidates) so formed are useful activated intermediates for pyrophosphate formation (177). The reaction involved is shown in Fig. 7. This method possesses the advantage that only one of the phosphorus atoms of the pair which goes to making the pyrophosphate is activated and this minimises the possibility of side reactions. When the other reactant is orthophosphate ($R_2$ = H in II, Fig. 7). The product is a nucleoside 5' pyrophosphate: other phosphomonoesters can be used however, (e.g. $R_2$ = glucose in II) and this method is frequently chosen for the synthesis of the nucleotide

FIG.7.

Reactions involved in the synthesis of pyrophosphate esters.

a) From a phosphorochloridate



I

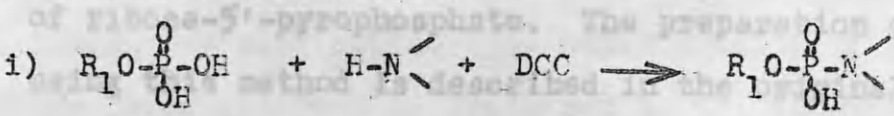
b) Using dicyclohexylcarbodiimide (DCC)

+ higher polyphosphates

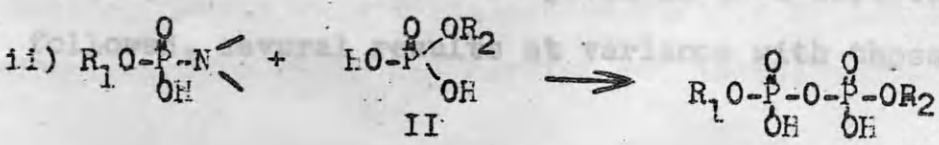
+ dicyclohexylurea

c) Via the phosphoramidate.

i)



ii)



II

pyrophosphate sugar derivatives.

The simplest method for the formation of nucleoside-5'-pyrophosphates, which was available when the present work was carried out is a modification of the original method in which dibenzylphosphorochloridate was used. This new method (169) obviates the need for protecting the second hydroxyl function of the nucleotide phosphate group. Michelson (169) found that by reacting dibenzylphosphorochloridate with the unblocked nucleotide under suitable conditions, high yields of the $P^1$-nucleosides-5' $P^2P^2$-dibenzylhydrogenpyrophosphate ($R_3$ = nucleoside, $R_4$ = H, $R_1$ = $R_2$ = benzyl in I, Fig. 7) were formed and these were easily converted to the nucleoside pyrophosphate by catalytic hydrogenation.

Because of its simplicity and high yield this method was used in the present work for the synthesis of uridine-5'-pyrophosphate and was adapted for the synthesis of ribose-5'-pyrophosphate. The preparation of UDP using this method is described in the original paper (169). Although the instructions given in this were carefully followed, several results at variance with those of

Michelson were observed; these are discussed in the section on synthesis of UDP.

The synthesis of ribose-5-pyrophosphate.

For reasons stated previously, the reported preparation of R5PP (176) using DCC was not used. An adaptation of Michelson's method, using dibenzylphosphorochloridate proved successful.

Ribose-5-phosphate synthesised in Expt. 8, which was 80% pure according to pentose analysis, was used in the preparation (Expt. 15). It was converted to the tri-n-octylammonium salt and this was reacted with dibenzylphosphorochloridate in the presence of an excess of tri-n-butylamine. The solvents, dioxan and benzene, were removed after reaction, and $P^1$-ribose-$5P^2P^2$-dibenzyl hydrogen phosphate was purified by precipitation from ether. The oil so obtained was hydrogenated in aqueous ethanol over palladised charcoal; uptake of hydrogen was very rapid. The calcium salts of phosphate esters were obtained as explained in the experimental section. These were fractionated further by ion exchange chromatography which was carried out in the cold-room

to minimise hydrolysis of the pyrophosphate. Details of the ion exchange procedure are given in the experimental section. Fractions were assayed for their pentose content. The elution pattern is shown in Fig. 8.

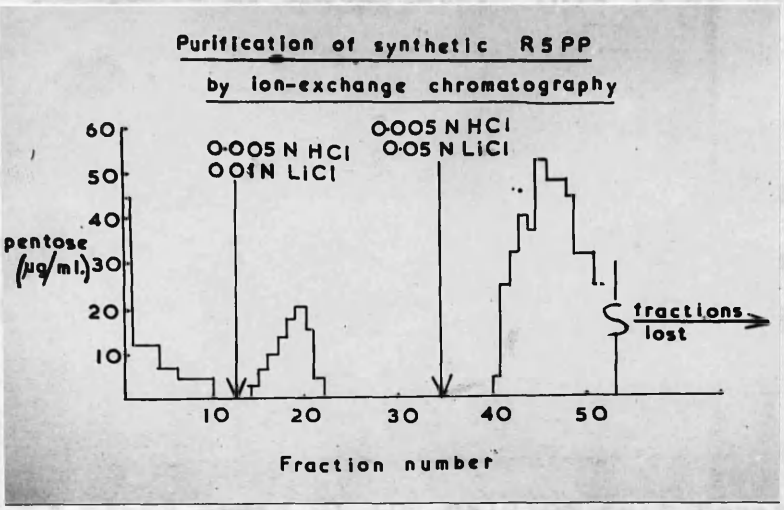
A break-down in the fraction collector caused a loss of some of the second (R5PP) peak but fortunately most of the substance appeared to have been eluted at this stage. Further losses may have arisen during the isolation of LiR5PP from excess LiCl. This latter was removed by washing the freeze-dried lithium salts with ethanol/acetone. The yield of the lithium salt (13mg. = 5.5% of theory) was lower than expected from the size of the peak. Phosphate analysis (G.M. 2) showed that the ratio of acid labile to total phosphate, 1:1·85, was as expected for R5PP.

The synthesis of uridine-5'-pyrophosphate.

The method described by Michelson (169) was followed exactly up to the calcium salt fractionation step and to this point it is similar to the preparation of R5PP described above.

FIG. 9

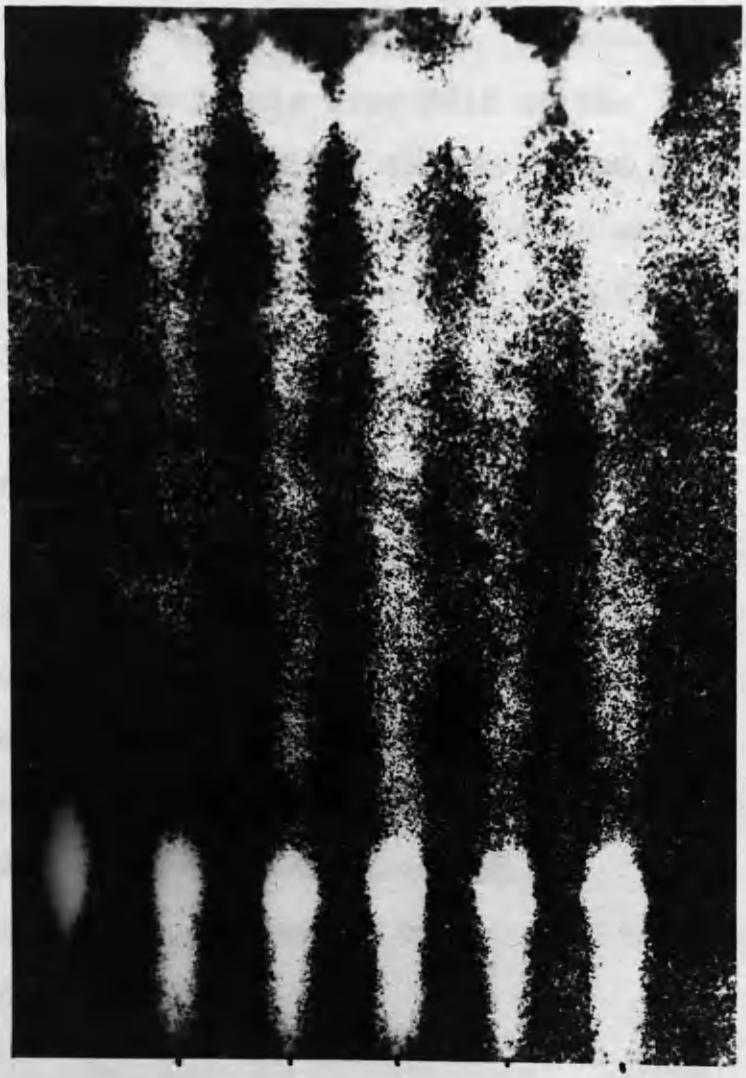
Ion-exchange chromatography of synthetic Ribose-5-pyrophosphate



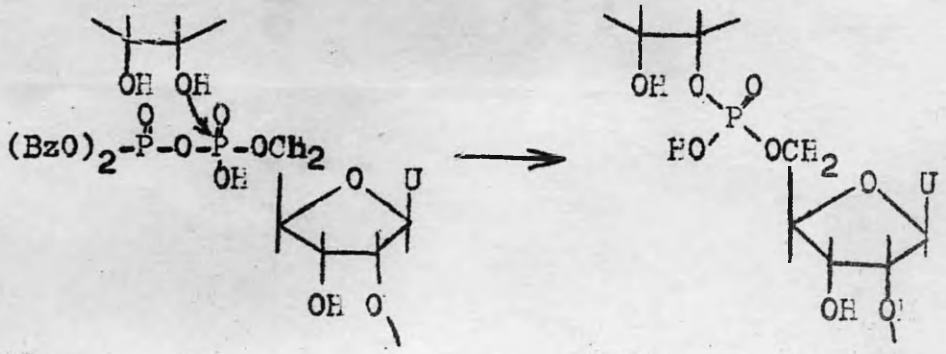
During the reaction of tri-n-octylammonium U5'P with dibenzylphosphorochloridate, samples were removed and applied to the origin of a paper chromatogram (Whatman No. 1). This was developed in Solvent 1 and is shown in Fig. 9. It is clear that after 3 hours reaction, conversion to $P^1$-uridine-5'-$P^2P^2$-dibenzylhydrogen pyrophosphate is incomplete. This is at variance with the observation of Michelson who found complete reaction at this stage. The gum precipitated from ether by petroleum was also applied to the chromatogram (last position on the right). This shows the presence of considerable amounts of unreacted U5'P. After hydrogenolysis of the benzyl groups, which was very rapid, the calcium salts were isolated as described by Michelson. A repeat of the first stage of the calcium salt fractionation used by this author, on a small scale showed that losses were high. The remainder of the product was therefore fractionated by ion exchange chromatography using the procedure described by Chambers (177). Uridine compounds were detected by measuring the optical density at 260mμ of the effluent fractions. The elution profile and the eluting solutions used are shown in Fig. 10. The first two peaks were eluted by salt and acid

# FIG. 9

## Reaction of U5'P and dibenzylphosphorochloridate



concentrations expected to elute U5'P and UDP respectively. When the fractions containing these two peaks were bulked and the total optical density at 260mμ of each peak (T O D ) determined it was found that for peak I $TOD_{260}$ = 4,517 and for peak II $TOD_{260}$ = 24,400. Together, these represent only a little over half of the TOD of the solution which was applied to the column (50,500 O.D. units) and it was clear that the rest must be accounted for by material which was not yet eluted from the column. Increasingly stronger solutions of salt and acid were applied until this material (peak III) was eluted. The conditions necessary to elute it (0.01N HCl, 0.20 N LiCl) suggest that the material consists of oligouridylic acid chains and this is supported by the fact that on paper chromatograms (Fig. 12) the material forms a streak from the origin. Michelson did not report the finding of these compounds. It is likely that their formation is due to an intermolecular phosphorylation between $P^1$-uridine-5'-$P^2p^2$-dibenzyl hydrogen pyrophosphate molecules:



Purification of uridine-5'-pyrophosphate by ion exchange Chromatography.

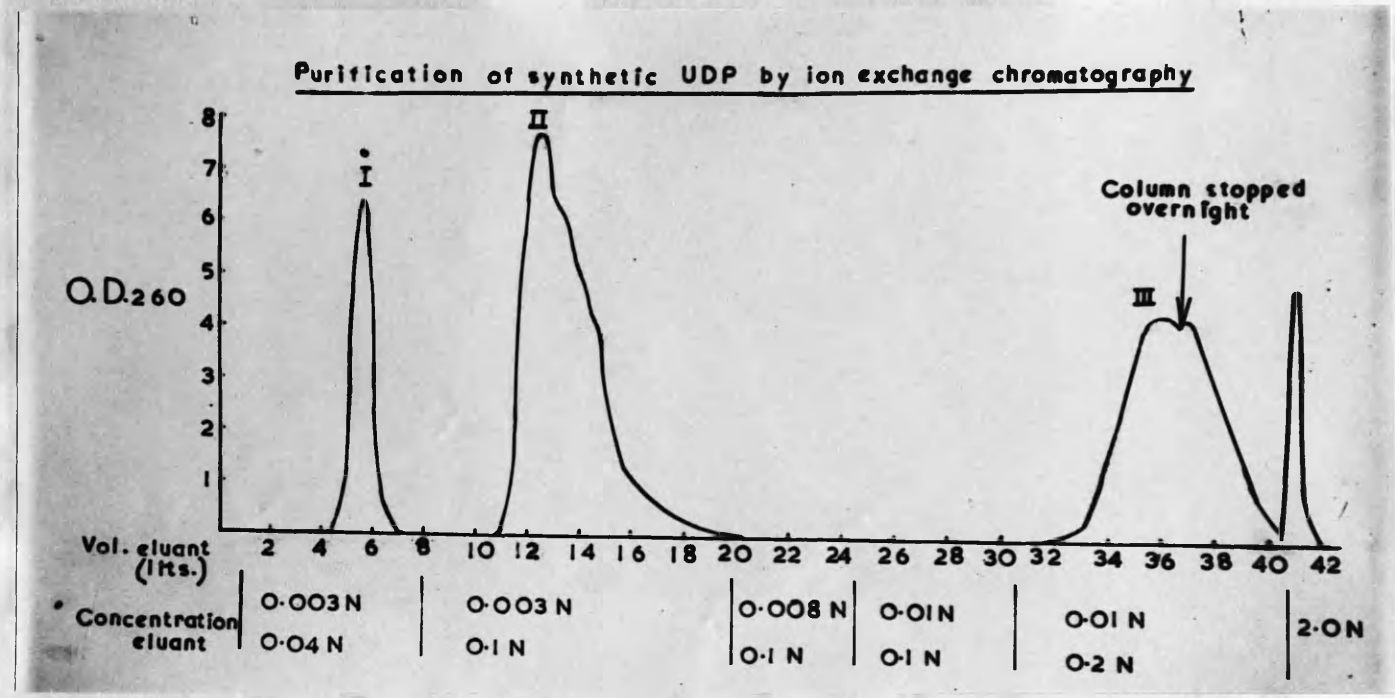
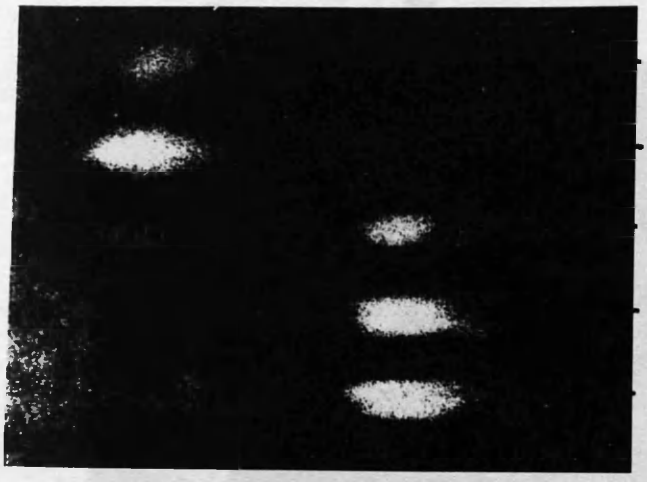



FIG. 10.

FIG. 11

Identification of nucleotides from ion exchange column by paper chromatography



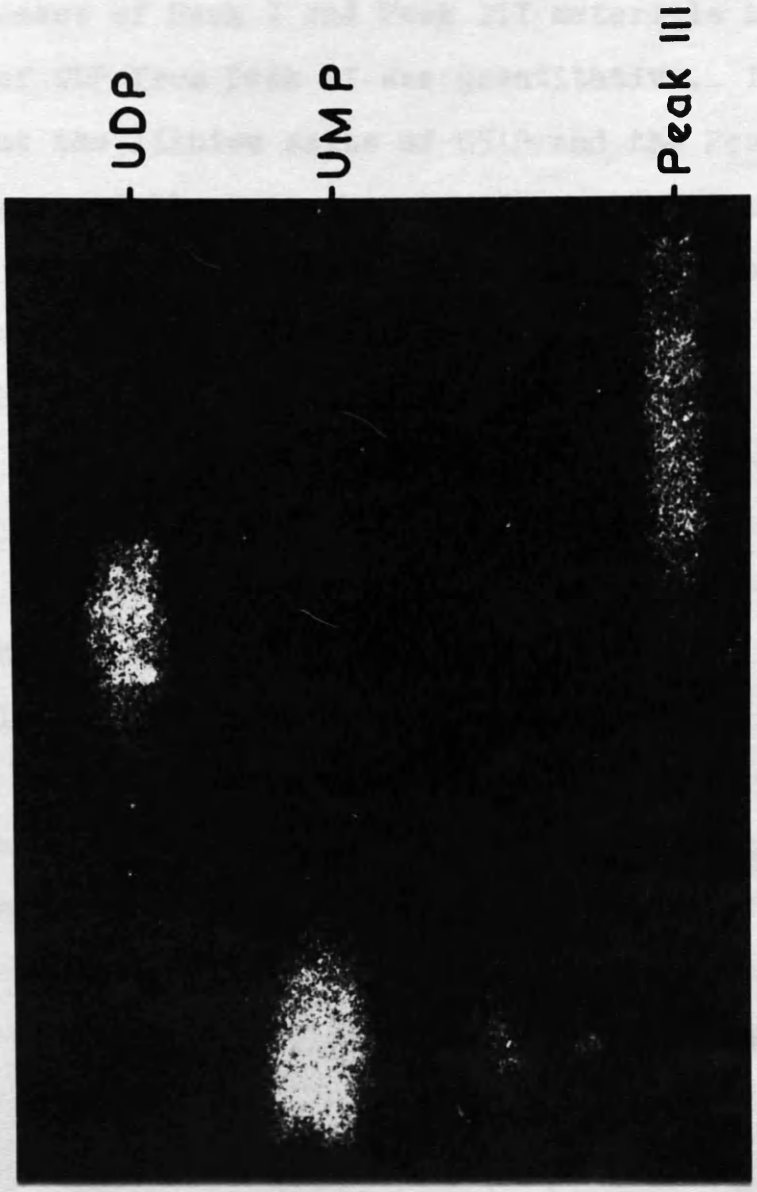
Authentic UMP
Peak I
Authentic UDP
Peak II
Peak II + UDP

direction of irrigation

Peak II + UDP
Peak II
Authentic UDP
Peak I
Authentic UMP

FIG. 12

## Paper chromatography of Peak III from ion exchange column



The lithium salts were isolated from the large excess of lithium chloride by washing the freeze-dried solids with anhydrous methanol (177). This resulted in high losses of Peak I and Peak III materials but recovery of UDP from Peak II was quantitative. It is likely that the lithium salts of U5'P and the Peak III material are soluble in methanol since a more recent technique for recovering lithium salts of phosphate esters involves precipitation from a solution in methanol by addition of acetone (178).

The final yield of lithium uridine-5'-diphosphate was 26%. Comparison of the $O.D._{260}$ with acid labile and total phosphate gave the molar ratio 1:1.33:2.3 (theory =1:1:2) for uracil:acid labile phosphate:total phosphate. The value for uracil was calculated assuming a molar extinction co-efficient of $9.7 \times 10^{-4}$ (California Foundation for Biochemical Research - "Properties of the nucleic acid derivatives"). The purity of the UDP according to extinction at 260mμ is 77%; according to phosphate determination, it is 95%.

Paper chromatograms in which materials from peaks I and II were compared with authentic U5'P and UDP are shown in Fig.11.

Preparation of 4,5-dihydrouracilriboside-5-pyrophosphate.

In order to circumvent the catalytic reduction of polyuridylic acid, which, as will be described in a later section, is often difficult, 4,5-dihydrouracilriboside-5'-pyrophosphate was prepared (Expt.17) to test the possibility of using it as a substrate for polynucleotide phosphorylase. Lithium UDP prepared as above was converted to the sodium salt and hydrogenated in slightly acid (pH4) solution, over 5% rhodium on alumina. The course of reaction was followed by measuring the optical density at 260mμ, which is destroyed by removal of the 4,5- double bond. When this had reached zero, the catalyst was filtered off, the pH of the filtrate was adjusted to 8 by the addition of caustic soda and the solution stored frozen at -18°C until required.

## SECTION II

## The enzymic and chemical synthesis of ribopolynucleotides. The partial purification of polynucleotide phosphorylase from E. coli.

The partial purification of polynucleotide phosphorylase from three bacteria - A. agilis (99), E. coli (179) and M. lysodiekticus (180) had been described when the present work was initiated. Both the A. agilis and E. coli enzymes appeared suitable for the purposes outlined previously, as they show a broad specificity towards the nucleoside diphosphates, which they convert into polynucleotides (with the reservation that GMP is incorporated very slowly from GDP). The M. lysodiekticus enzyme, on the other hand, when purified by a method involving trypsin digestion (180) was specific for the polymerisation of ADP and therefore appeared unsuitable.

Because the correct strain of E. coli was available ( a generous gift of Dr. A. Berrie who obtained it as a freeze-dried preparation from the U.S. Type Culture Centre) and because the purification procedure appeared easier than that for the enzyme from A. agilis, E. coli was chosen as a source of the enzyme.

Growth of E. coli and extraction of protein.

Precautions were taken in all work with the bacterium, to avoid contamination with other organisms. Thus, growth media and vessels were sterilised by autoclaving before use and transfer of the bacterium during inoculation was made under aseptic conditions. In addition, microscopic examination of the cultures was used to check for contamination (for help in this work, the author is indebted to Dr. R.B. Morrison). The cells were grown as described by Littauer and Kornberg (179) (G.M. 10) and harvested at room temperature in a continuous-flow centrifuge.

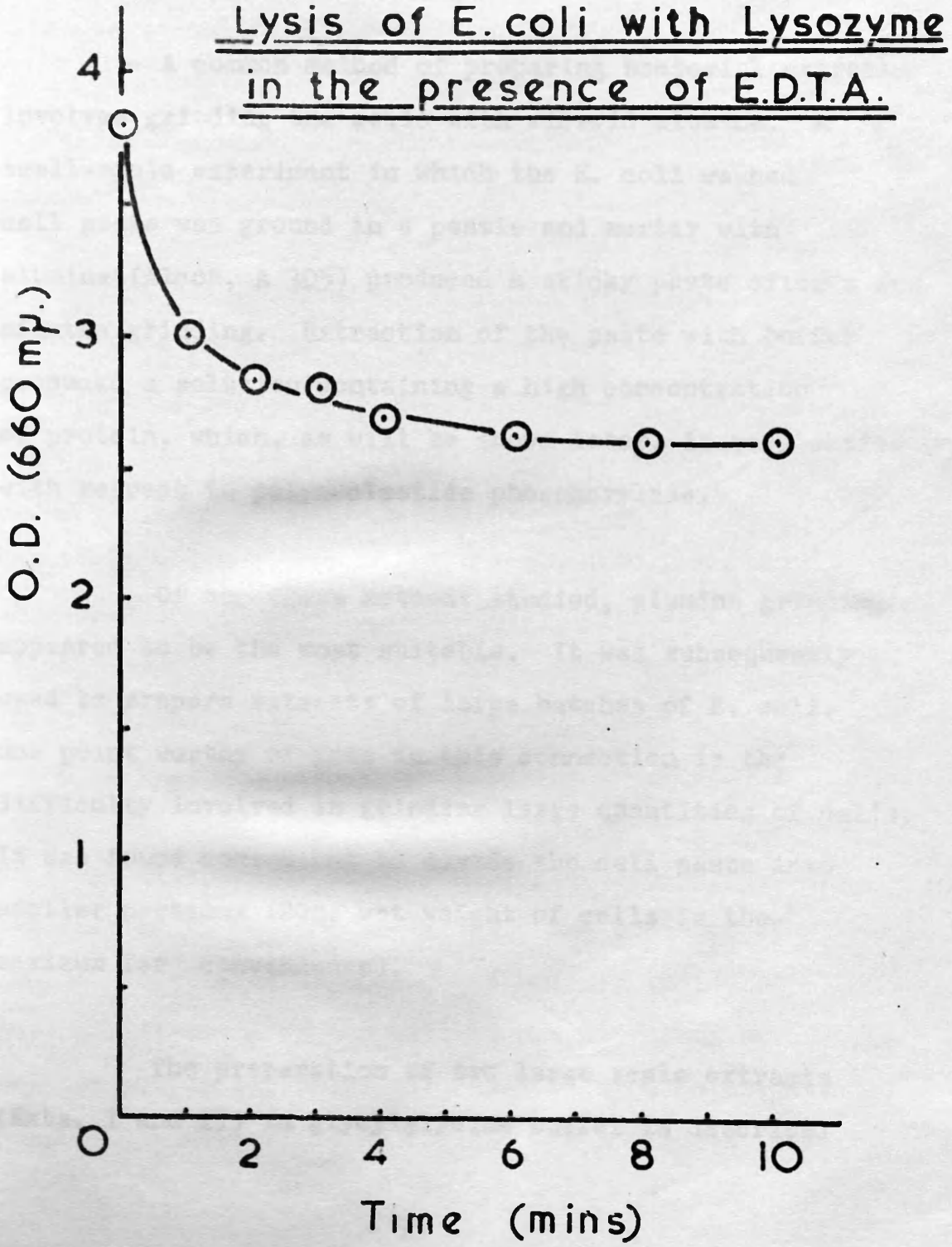
In the preparation described by Littauer and Kornberg cells were disrupted in dilute suspension by subjecting them to treatment with a 10Kc. ultrasonic oscillator. Such a machine, but of lower power (9Kc.) was made available to the author by the courtesy of Dr. R.B. Morrison. With the smaller instrument disintegration is performed on concentrated cell pastes in small vessels and it was not possible therefore, to calculate the conditions necessary to achieve the fractionation described by Littauer and Kornberg.

This fractionation depended, rather critically, upon a preliminary disruption of the cells by a 10 min. period in the disintegrator after which the particulate matter, containing the activity was centrifuged down and re-extracted for a further 30 min. in the disintegrator. Using the 9Kc. apparatus, on a concentrated cell paste, cell rupture was not complete, as judged by microscopic examination, after 30 min. (Expt. 18).

Two other methods of cell disruption were tested as alternatives to ultra-sonic disintegration.

Repaske (181) has described the use of lysozyme in the preparation of extracts from Gram-negative bacteria, which are disrupted by lysozyme in the presence of EDTA. Incubation of a dilute cell suspension with lysozyme in the presence of EDTA did cause some dissolution of the cell walls. This was clear from the microscopic appearance of the treated cells and from the initial rapid fall in optical density at 660mμ to 66% of the original value (Fig. 14). However, these observations also showed that lysis of the cell wall was not sufficient to cause the cell contents to be

FIG. 14


Lysis of E. coli with Lysozyme
in the presence of E.D.T.A.
O.D. (660 mμ.)
4
3
2
1
0
2
4
6
8
10
Time (mins)

emptied into the surrounding medium.

A common method of preparing bacterial extracts involves grinding the cells with microid alumina. A small-scale experiment in which the E. coli washed cell paste was ground in a pestle and mortar with alumina (Alcoa, A 305) produced a sticky paste after a few minutes grinding. Extraction of the paste with buffer produced a solution containing a high concentration of protein, which, as will be shown later, is very active with respect to polynucleotide phosphorylase.

Of the three methods studied, alumina grinding appeared to be the most suitable. It was subsequently used to prepare extracts of large batches of E. coli. One point worthy of note in this connection is the difficulty involved in grinding large quantities of cells. It was found convenient to divide the cell paste into smaller portions (20g. wet weight of cells is the maximum for convenience).

The preparation of two large scale extracts (Exts. I and II) in glycylglycine buffer is described

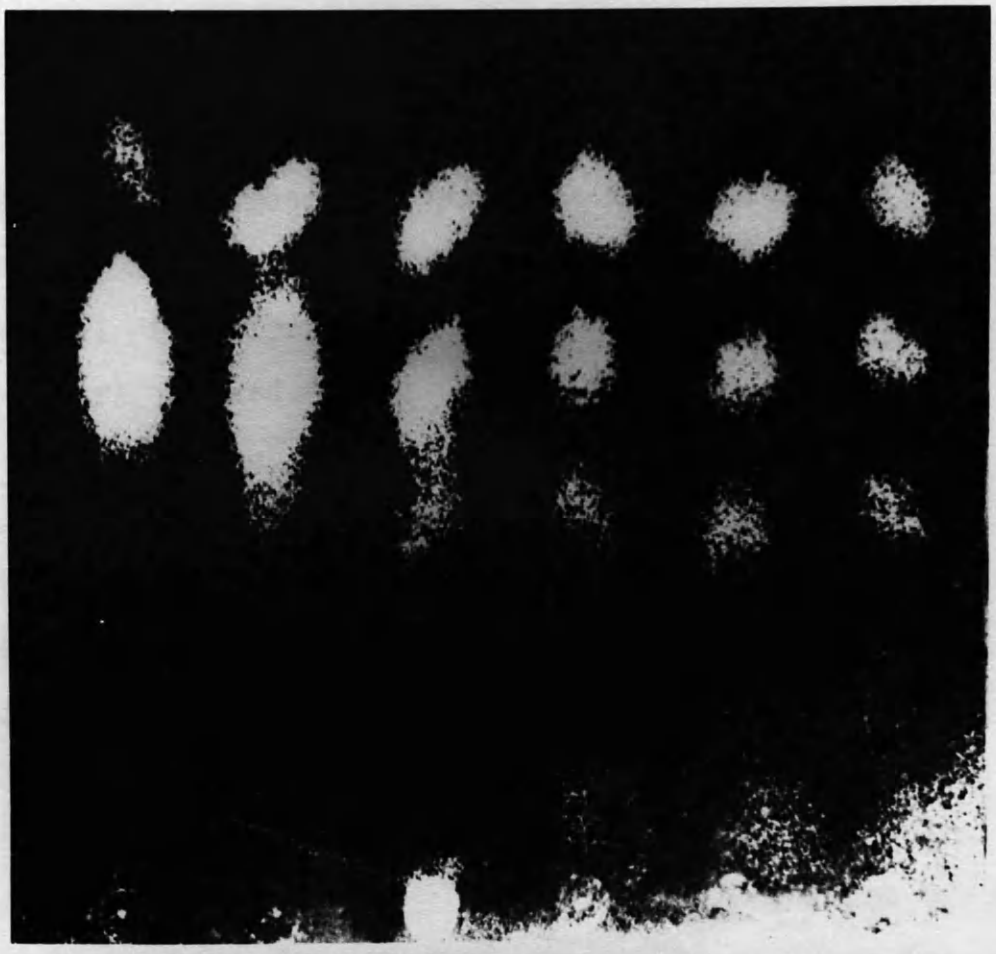
in Expt. 20. These extracts were used in all subsequent attempts to purify the polynucleotide phosphorylase.

Demonstration of polynucleotide phosphorylase activity in crude extracts of E. coli.

Before proceeding with attempts to purify the enzyme, it was required to show that crude extracts obtained by grinding E. coli cells with alumina did in fact contain polynucleotide phosphorylase. A small digest, containing a high concentration of ADP was prepared (Expt. 21). Aliquots were withdrawn at intervals and boiled to stop the reaction. The denatured protein was centrifuged and the supernatant solution applied to the starting line of a paper chromatogram. This was developed in Solvent 3 and is shown in Fig. 15. The ½ hr. digest shows a strong spot at the origin, indicating formation of polynucleotide. This has disappeared after 1 hr., presumably through degradation by phosphodiesterase. The presence of enzymes capable of degrading the polymer indicated that purification of the extract was necessary before the polynucleotide phosphorylase could be used to prepare polyU. The appearance of AMP and ATP indicates the

FIG. 15

## Paper chromatogram showing action of crude E.coli extract on ADP



presence of an adenylate kinase.

The measurement of polynucleotide phosphorylase activity.

The reaction catalysed by polynucleotide phosphorylase:

$$n\ X\text{-}R\text{-}P\text{-}P \rightleftharpoons (X\text{-}R\text{-}P)_n + nPi$$

presents several products which can be measured to estimate the extent of reaction and hence the activity of the enzyme. Most commonly, the substrate used in the assay is ADP. Less frequently, the phosphorolytic reaction on polyA is used.

Because the reaction is reversible, incubation of the enzyme with ADP in the presence of radio-active orthophosphate, causes an exchange between this and the terminal phosphate group of the ADP:

$$A\text{-}P\text{-}P + Pi^{32} \rightleftharpoons A\text{-}P\text{-}P^{32} + Pi$$

The radioactive ADP can be measured after absorption on charcoal. This technique was first described by Grunberg-Manago et al. (99) and is now the most frequently used method for the assay of polynucleotide phosphorylase.

The formation of polyA from ADP is accompanied

by the release of an equivalent amount of inorganic phosphate Measurement of phosphate release presents a convenient means of estimating the extent of reaction (99).

Another method of estimating the amount of polymer formation depends upon the acid insolubility of polyA. After reaction with $C^{14}$-ADP the polyA produced can be measured from its radioactivity (179). Alternatively, with non-radioactive ADP the polymer can be measured by dissolving it and estimating the absorption at 260mμ.

The phosphorolysis of polyA can be followed by measuring the incorporation of radioactive orthophosphate into material which is adsorbed by charcoal (179):

$$\text{polyA} + n\text{Pi}^{32} \rightleftharpoons n\ \text{A-P-P}^{32}$$

Alternatively, the ADP produced from polyA can be measured (98) through coupling with the reactions catalysed by pyruvic kinase and lactic dehydrogenase. This system finally produces oxidised co-enzyme I ($DPN^+$) equivalent to the amount of ADP produced by the polynucleotide phosphorylase. $DPN^+$ is estimated from the decrease in absorbency at 340mμ due to removal of reduced co-enzyme I (DPNH).

All of the assay methods described above are subject to interference from other enzymes commonly present in bacterial extracts. This is illustrated by the following hypothetical cases. If polynucleotide phosphorylase activity is estimated from the formation of acid insoluble polymer, the presence of enzymes which break down this product (e.g. nucleases, phosphodiesterases) will give an artificially low result. Assay methods which depend on the release of inorganic phosphate suffer from interference by phosphatases which, in conjunction with adenylate kinase, can form this product by a different route:

$$2\ ADP \xrightarrow{\text{kinase}} ATP + AMP \xrightarrow{\text{phosphatase}} adenosine + Pi$$

This would lead to an artificially high estimate of polynucleotide phosphorylase activity.

It has been pointed out by Ochoa and Mii (98) that assay methods based on the phosphorolysis reaction are less open to error than those which make use of the synthetic reaction. However, the scarcity of the requisite polyA precluded this as a routine assay, in the present

work, and a method based on the release of inorganic phosphate from ADP was used throughout. This method (G.M.11) differs from published procedures and provides a rapid convenient measurement of activity such as is required during the purification of an enzyme. The ADP is dissolved in Tris buffer containing magnesium chloride. It is convenient to prepare the buffer solution in quantity since it can be stored indefinitely over chloroform. ADP solution is prepared as required, since storage of solutions containing ADP, even in a frozen state, leads to appreciable formation of inorganic phosphate after a few weeks. The test is carried out by mixing equal volumes (0.1 ml. is convenient) of the enzyme solution and the ADP solution in a conical centrifuge tube. After a prescribed digestion period at 37°C., protein is precipitated by the addition of perchloric acid (approx. 5ml.). The method used to remove the protein depends on the form of the precipitate. Crude protein preparations, rich in nucleic acid, usually coagulate into large clots on addition of the acid and these are most conveniently removed by filtering through a small glass-wool plug. Purer enzyme preparations give a disperse precipitate

which is more easily removed in the centrifuge. In either case, the precipitate is washed with more perchloric acid and the washings are combined with the first filtrate or supernatant solution. For rapid, routine work, the final volume can be taken as the sum of the two portions of perchloric acid, ignoring the small losses. Greater accuracy is achieved by adjusting the final volume of the supernatant acid, in a graduated tube.

The inorganic phosphate produced is estimated directly by adding the molybdate reagents (G.M. 2, omitting the perchloric acid) and measuring the blue colour produced.

To express enzyme activity, the system adopted by Littauer and Kornberg (179) has been used throughout the present work. The unit of activity is that amount of enzyme which will release one micromole of inorganic phosphate from ADP under the conditions of the assay. Specific activity (S.A.) is expressed as units per milligram of protein.

Protein concentration was measured by two

methods. When the concentration was high, a quantitative biuret test (182) (G.M. 12a) was used; for dilute solutions the more sensitive method of Lowry et al. (183)(G.M. 12b) was employed. Both methods were standardised using crystalline bovine serum albumin but with the same sample of E. coli protein, the two methods frequently gave results which differed appreciably (as much as 100% in extreme cases). No attempts were made to correlate the results using these methods. However, when a direct comparison of protein concentration was essential only one method was used.

Some observations on the assay of polynucleotide phosphorylase by phosphate release.

It is relevant at this stage to discuss some faults of the phosphate release assay method which emerged during work on the fractionation of polynucleotide phosphorylase preparations.

It should be said in favour of the method, that there appears to be no interference from phosphatase activity, in the case of E. coli extracts, of the type mentioned in the "hypothetical case" discussed earlier.

This is clear from the result shown in Fig.15. If an interfering phosphatase had been present then adenosine would have appeared on the chromatogram.

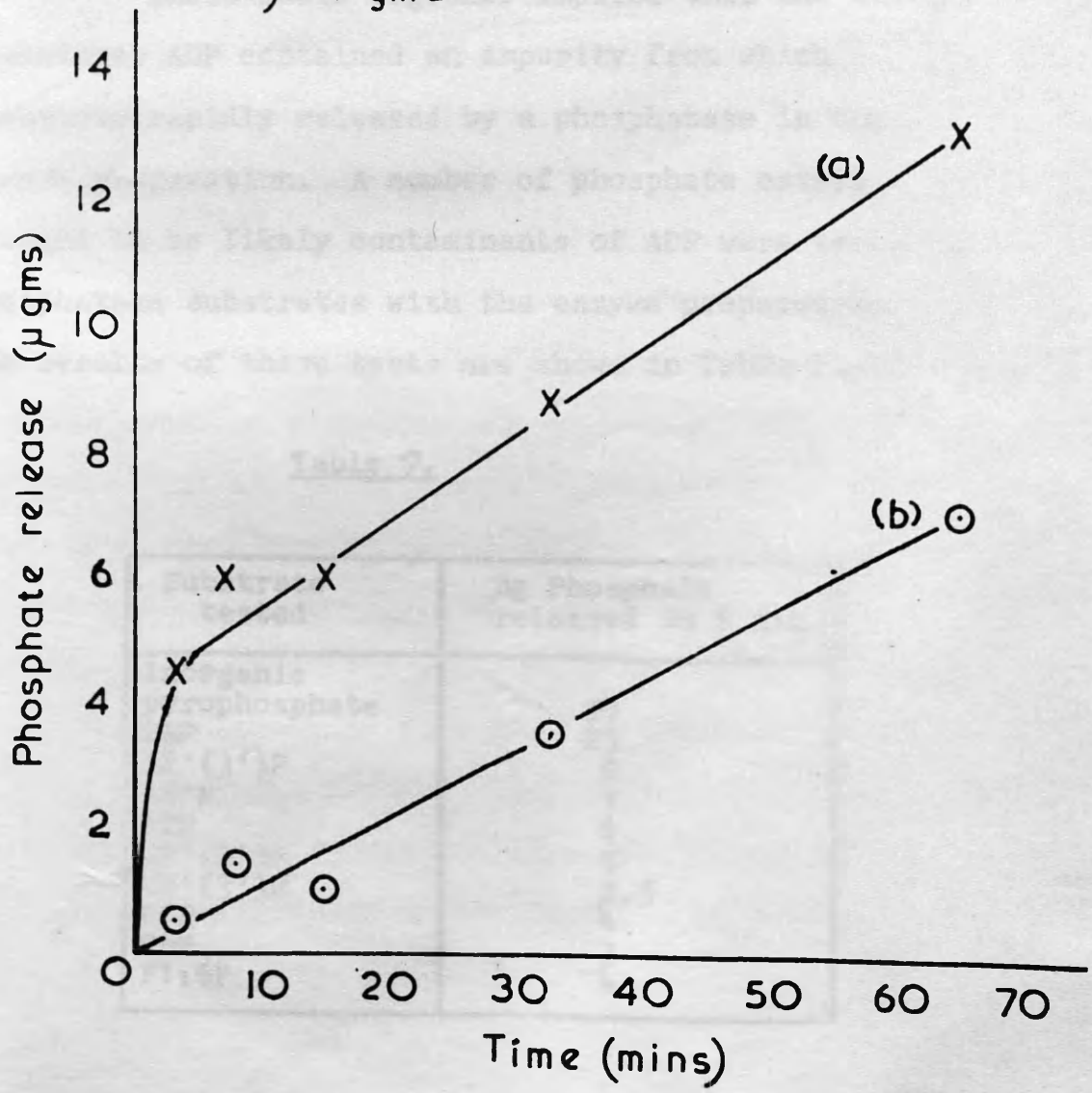
An error of this type did arise because of the presence in one commercial sample of ADP (obtained from Biochemica Boehringer AG.) of an impurity which was later shown to be inorganic pyrophosphate. Anomalous results were caused by the rapid release of inorganic orthophosphate from this ADP by a highly active inorganic pyrophosphatase which is present in the enzyme preparations. The results of two types of experiment led to the discovery of this error. Attempts were made to concentrate certain enzyme preparations which apparently contained polynucleotide phosphorylase activity but were of low protein concentration. Several methods were successful in concentrating the protein, but in each case the release of inorganic phosphate from ADP remained exactly the same. Secondly, a progress curve of the release of inorganic phosphate using the same enzyme preparation (Fig.16a) showed a rapid initial release of phosphate which was then followed by a much slower release. A progress curve using the same enzyme preparation with a different sample of ADP

FIG. 16

Release of inorganic phosphate from two commercial samples of A.D.P.

a) Boehringer & sons

b) Sigma



(obtained from Sigma Chemical Company) is shown in Fig. 16b. It can be seen that there is no preliminary rapid release of Pi as with the Biochemica Boehringer ADP and further that the rate of phosphate release corresponds with the second phase of the release from Boehringer ADP.

These facts together implied that the Biochemica Boehringer ADP contained an impurity from which phosphate rapidly released by a phosphatase in the enzyme preparation. A number of phosphate esters thought to be likely contaminants of ADP were tested as phosphatase substrates with the enzyme preparation (Expt. 23). The results of these tests are shown in Table 7.

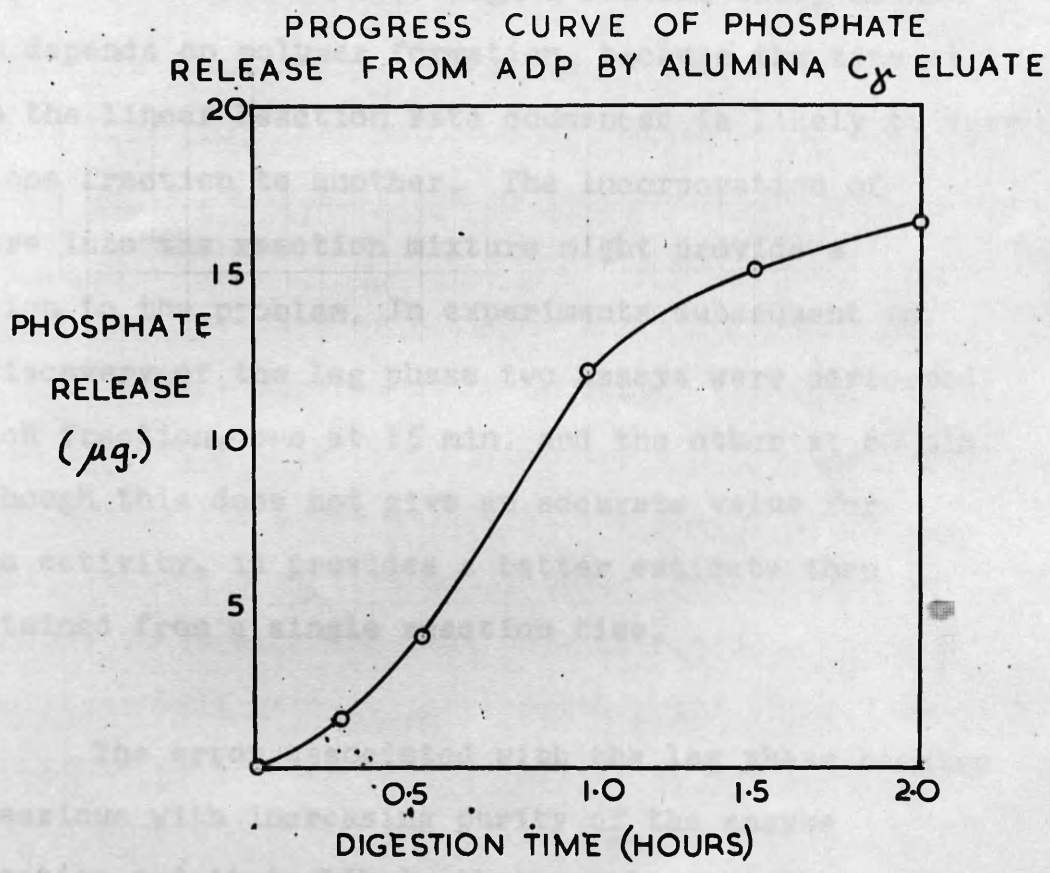
Table 7.

| Substrate tested | µg Phosphate released in 5 min. |
|---|---|
| inorganic pyrophosphate | 50 |
| PAP | 23 |
| A2'(3')P | 0 |
| A5'P | 1 |
| ATP | 1 |
| C2'(3')P | 5 |
| U2'(3')P | 2.5 |
| G1P | 4 |
| G6P | 3 |
| F1:6P | 4 |

From these results it appears that the most likely source of the extra, rapid phosphate release with Biochemica Boehringer ADP is either inorganic pyrophosphate or adenosine-2'(3')-5'-diphosphate (PAP). Evidence in favour of the former is the observation that after all adenine containing substances had been removed from the ADP by absorption on charcoal, the substrate for the phosphatase remained (Expt. 24). In fact, a similar experiment with PAP shows that the rapid phosphate release from this compound by the enzyme preparation is due to an inorganic contaminant. Experiments which were assayed using the impure sample of ADP have not been included in the ensuing discussion unless they serve to illustrate the anomalous results obtained with this ADP or unless there was evidence that the phosphate release was due at least in part to polynucleotide phosphorylase. Since the amount of inorganic pyrophosphate in each aliquot of the assay mixture is known fairly accurately it can be taken into account by subtracting from the total phosphate release. Where such results are included, reference is made to the fact that they were obtained using the sample of ADP (hereafter referred to as contaminated ADP).

Other anomalous estimations of enzyme activity may have occurred because of a lag phase in the reaction. At the outset of this work a lag phase had recently been demonstrated for highly purified preparations of the A. agilis enzyme [300-fold purified (184)] which were low in polynucleotide. However, for the E. coli enzyme which had been purified 170-fold and was also free from polynucleotides (179), no lag phase had been reported for any of the reactions of the enzyme. Evidence will be presented later, that E. coli extracts only partially purified (tenfold or less) and which contain appreciable amounts of polynucleotide show a lag phase in the release of inorganic phosphate from ADP and UDP. This characteristic of the enzyme reaction was discovered when digestion times longer than the customary 15 or 30 min. were used in the assay of purified fractions. Using these longer assay times, the enzyme preparations showed an unexpectidly high activity. A full progress curve of the reaction (Fig. 17) shows that there is, in fact, a short but significant lag phase, which would lead to a serious underestimation of the enzyme activity using the shorter digestion times.

FIG. 17


PROGRESS CURVE OF PHOSPHATE RELEASE FROM ADP BY ALUMINA Cγ ELUATE
PHOSPHATE RELEASE (μg.)
20
15
10
5
0·5
1·0
1·5
2·0
DIGESTION TIME (HOURS)

This makes it likely that in some of the experiments described here fractions which contained an appreciable concentration of the enzyme were discarded as inactive because the phosphate release was measured during the lag phase. In the light of the existence of the lag phase, it is difficult to design a routine assay method which depends on polymer formation, because the time at which the linear reaction rate commences is likely to vary from one fraction to another. The incorporation of primers into the reaction mixture might provide a solution to the problem. In experiments subsequent to the discovery of the lag phase two assays were performed on each fraction, one at 15 min. and the other at 60 min. and though this does not give an accurate value for enzyme activity, it provides a better estimate than is obtained from a single reaction time.

The error associated with the lag phase becomes more serious with increasing purity of the enzyme preparation and it is likely that results obtained with the cruder extracts are reasonably reliable.

Preliminary experiments on the purification of polynucleotide phosphorylase from extracts of E. coli.

For the purpose of synthesising polyU, an active enzyme preparation as free as possible from inactive components is desirable. The contaminants present in the crude extract, which are most likely to cause difficulties are RNA, which would prove difficult to remove from polyU and ribonuclease which would destroy the polyU as it is synthesised by polynucleotide phosphorylase.

From the data of Littauer and Kornberg,(179) the E. coli enzyme appears to be an acidic protein. Thus, it is precipitated with protamine and travels rapidly towards the anode during electrophoresis. Ribonucleases are most frequently basic proteins and it is therefore to be expected that polynucleotide phosphorylase will have an isoelectric point which lies between those of RNA and RNase. Advantage has been taken of this in absorption and precipitation procedures designed to remove these two contaminants. Where possible, experiments with the enzyme solutions were carried out in vessels cooled in ice-water baths.

Prolonged experiments were performed in a cold-room at 4°C.

Initially, observation of the effect of the precipitant or absorbant on the polynucleotide phosphorylase activity and total protein concentration were taken as indications of the usefulness of the procedure. A method was judged successful if it removed inactive protein or RNA without seriously reducing the polynucleotide phosphorylase activity. If a procedure quickly reduced the polynucleotide phosphorylase activity it was not considered useful.

Nucleic acids are readily removed from native protein by precipitation as the manganese salt (185). This procedure was employed in the original preparation of polynucleotide phosphorylase from E. coli by Littauer and Kornberg. After removing the precipitated nucleic acids, these workers subjected the supernatant solution to dialysis and precipitated the activity by the addition of protamine. An attempt to reproduce this procedure with the E. coli extract prepared by grinding with alumina is described in Expt. 25. The protein concentration of this extract is, of course, much higher than the sonic

extract used by Littauer and Kornberg. The specific activity, however, is approximately the same. In Table 8 below are shown the results of assaying the crude extract, the manganese supernatant solution after dialysis and the supernatant solution after removing the protamine precipitate. A second addition of protamine ccaused no further precipitation.

Table 8.

| Protein | Concentration of protein - mg/ml | Phosphate release in 15 min. (μg) | S.A. |
|---|---|---|---|
| Crude extract | 20.5 | 7.25 | .93 |
| Mn sup. | 18.5 | 4.5 | .65 |
| Protamine sup. | 9.8 | 3.0 | .41 |

The failure of the protamine to precipitate the activity discouraged further work with this method. The precipitation of nucleic acids using manganese appeared, from these results, to cause, a considerable loss of activity. It is possible that the loss occurred during dialysis. In later experiments (with Ext. II) where a high concentration of phosphate in the extract made a dialysis step necessary, the manganese precipitation was used more successfully.

The acidic earth, bentonite, has been used as an adsorbent for ribonuclease by Brownhill, Jones and Stacey. They found (186) that, during the isolation of RNA from yeast, degradation by RNase could be prevented if the material were extracted in the presence of bentonite. A preparation of a standard suspension of the clay described by these authors (G.M. 13) was carried out, with the modification that at the final stage, water instead of acetate buffer was used to make up the suspension. To determine the effect of adding bentonite on the activity and protein concentration of the crude E. coli extract, (Ext. II), which had been dialysed against 0.9% KCl for 18 hrs. a series of identical aliquots of the crude extract was treated with bentonite suspension (Expt. 26). The amounts of the suspension used varied from 0.2 to 1.0 x the volume of the protein solution. After the suspension had been thoroughly mixed with the protein solution, the volume was made up to double the original volume of protein solution and the bentonite removed by centrifugation. Aliquots of the supernatant solution were removed and assayed for polynucleotide phosphorylase (G.M. 11), RNase (G.M. 14) and protein (G.M. 12b)

The results are illustrated in Fig.18 (Table 9) in which the two enzymic activities, the protein concentration and the specific activity of polynucleotide phosphorylase are plotted as ordinates against the amount of bentonite used, as abscissa. They show that polynucleotide phosphorylase and inert protein are removed at approximately the same rate, while RNase is totally removed by the first two additions of bentonite. This method of removing traces of RNase from polynucleotide phosphorylase extracts would appear to be very useful and it has been employed in later experiments with partially purified extracts.

The removal of polyribonucleotides from DNA by using charcoal has been described by Zamenhof et al. (186). A standard suspension of Norit A was prepared according to the method described by these authors (G.M. 15) and aliquots of the crude extract were treated with 0.1 to 0.6 vols. of the suspension in a manner similar to that used for the bentonite treatment. Results of polynucleotide phosphorylase assay of the supernatant solutions after removing the charcoal, are

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FIG. 18

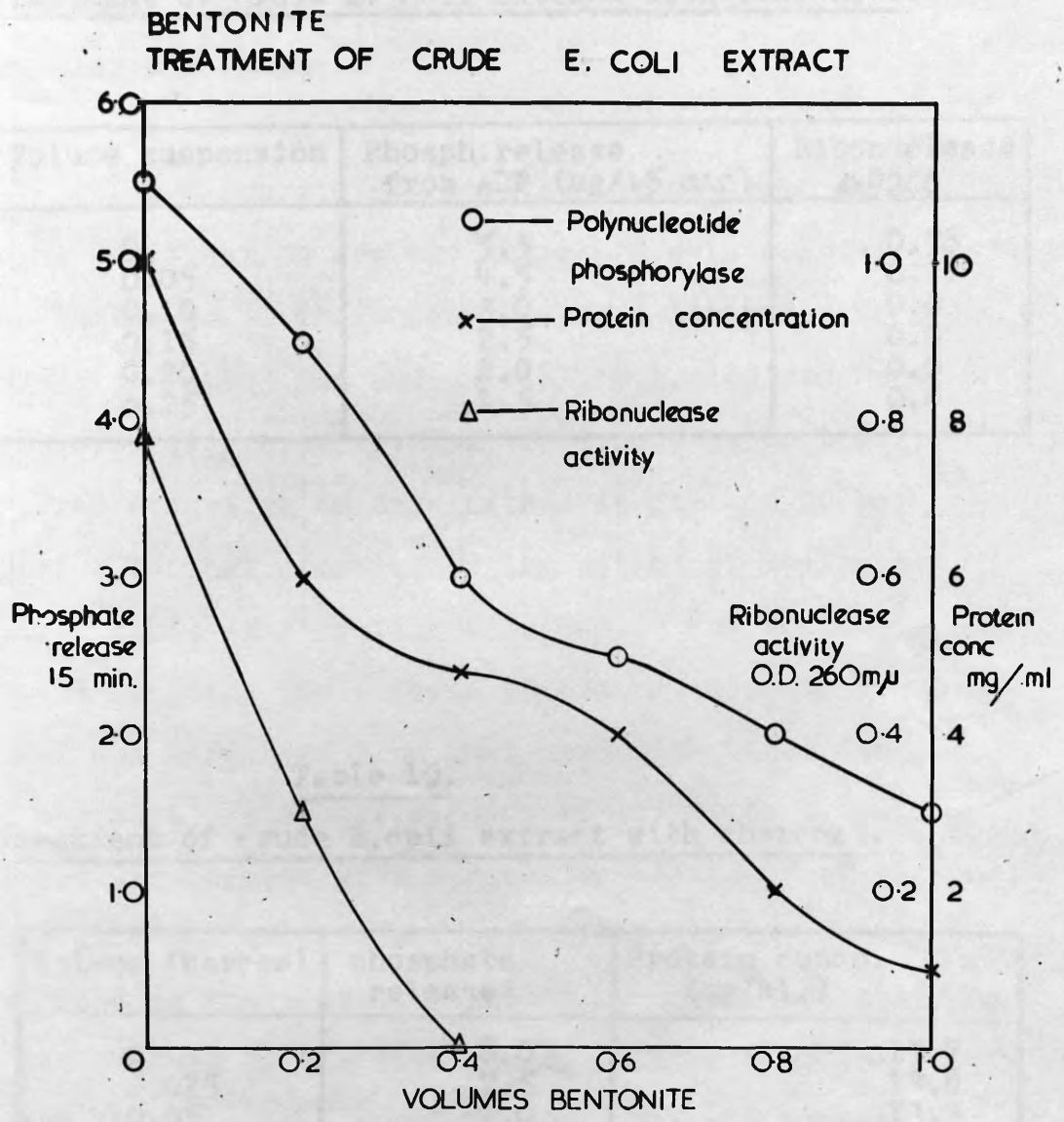

BENTONITE
TREATMENT OF CRUDE E. COLI EXTRACT
Polynucleotide phosphorylase
Protein concentration
Ribonuclease activity
Phosphate release 15 min.
Ribonuclease activity O.D. 260mμ
Protein conc mg/ml
6·0
5·0
4·0
3·0
2·0
1·0
0
1·0
0·8
0·6
0·4
0·2
10
8
6
4
2
0·2
0·4
0·6
0·8
1·0
VOLUMES BENTONITE

Table 9

Treatment of crude E. coli extract with bentonite.

| Volume suspension | Phosph.release from ADP (ug/15 min) | Ribonuclease ΔD260 |
|---|---|---|
| 0 | 5.5 | 0.78 |
| 0.05 | 4.5 | 0.31 |
| 0.10 | 3.0 | 0.0 |
| 0.15 | 2.5 | 0.0 |
| 0.20 | 2.0 | 0.0 |
| 0.25 | 1.5 | 0.0 |

Table 10.

Treatment of crude E.coli extract with charcoal.

| Volume charcoal | phosphate release | Protein concn. (mg/ml.) |
|---|---|---|
| 0 | 8.0 | 15.7 |
| 0.025 | 7.5 | 13.8 |
| 0.05 | 7.0 | 13.3 |
| 0.10 | 5.0 | 12.8 |
| 0.15 | 4.0 | 11.5 |

shown in Table 10. It is evident that the enzyme was adsorbed much more readily than the inert protein and it appeared unlikely that this method would be useful for purifying the enzyme.

Two protein adsorbents commonly used in enzyme purification are the inorganic gels calcium phosphate and alumina Cγ. These were prepared according to the methods of Tsuboi and Hudson (187, G.M. 16) and Bauer (188, G.M. 17), respectively. Calcium phosphate gel prepared according to this method is claimed to be superior to that prepared by the method of Keilin and Hartree (188) as a protein adsorbent. The method adopted to study the effects of adding increasing amounts of gel was different from that used with bentonite and charcoal. A single aliquot of the crude E. coli extract was treated with successive additions of the gel. After each gel aliquot had been added and thoroughly dispersed in the protein solution by mechanical stirring it was removed in the centrifuge. Aliquots of the supernatant solution were taken for activity and protein assay, and the remainder was treated with another

aliquot of the gel. Before the gel was added to the protein solution it was packed into a loose pellet by centrifugation. In spite of this the protein solution was diluted considerably at the end of the experiment, by water from the gel. The dilution of the protein solution by this water was not taken into account in the results.

Experiment 28 describes the treatment of crude Ext. I with calcium phosphate. The results (Table 19.) show that the first additions of calcium phosphate caused an increase in the phosphate release by the enzyme. This result has been observed in other experiments with this adsorbent and it is likely that it is due to the removal of an enzyme inhibitor. With subsequent additions the polynucleotide phosphorylase is removed in approximately the same proportion as the total protein.

Elution of the gel fractions bearing the highest concentration of activity, with sodium citrate, gave very high blank values in the phosphate determinations and this is no doubt due to the solubilisation of the

gel by citrate. Elution with ammonium sulphate gave rise to solutions of very low polynucleotide phosphorylase activity. It appeared that this adsorbent was unsatisfactory for purification of the crude extract.

Preliminary experiments with alumina C$\gamma$ showed that this was more active in adsorbing the polynucleotide phosphorylase from crude extracts of E. coli than the calcium phosphate gel and furthermore, early experiments with sodium bicarbonate as an eluting agent provided solutions of high enzyme activity. Several experiments were carried out using alumina C$\gamma$ and a standard procedure was devised which consistently provided an active enzyme. The experiments which led to this method of purification, together with an account of some attempts to purify the alumina eluate further are described in the following paragraphs.

<u>The partial purification of polynucleotide phosphorylase on alumina C$\gamma$ and further treatment of the enzyme.</u>

In all, 4 large scale experiments (Expt. 29a,b,c,d) using alumina C$\gamma$ on Ext. I were carried out. In two

experiments Ext. II was used.

The adsorption step was always performed by adding the alumina in at least two portions, since early observations indicated that the first addition removes considerable inactive protein and only a little of the polynucleotide phosphorylase. The first portion of gel was therefore discarded. Further additions of the gel adsorbed most of the activity and a similar proportion of the total protein.

No pretreatment of the crude extract was applied to Ext. I before addition of the alumina Cγ. For reasons discussed later, however, Ext. II was pretreated and this led, fortuitously, to an improvement in the preparation.

The experiments with Ext. I also differed from later experiments in the elution procedure. In early experiments the gel fraction(s) which had adsorbed most enzyme were dispersed, by high-speed stirring, in the eluting buffer. The suspension was allowed to stand in

the refrigerator before a final mixing, after which the gel was separated in the centrifuge. The process was repeated until the eluted fractions were almost devoid of activity. The results of four experiments are shown in Tables 11-14. Experimental details not tabulated with these results are to be found in the corresponding experimental section.

The results of Expt. 29d in which the ADP sample contaminated with inorganic pyrophosphate was used for the assay, will be discussed later. In the general discussion of the results of these experiments this particular case is ignored.

The results in Expts. 29a,b,c, show that sodium bicarbonate is slightly more effective as an eluting agent than sodium citrate. Phosphate, which is the customary eluting agent, was not used because of its interference with the assay method.

The purification achieved by this step is approximately threefold for the combined best fractions.

## Table 11.

Fractionation of E. coli Ext. 1 on alumina Cγ (Expt. 29a).

| Protein Fraction | Vol. gel (ml) | Vol. Protein soln. (ml) | Pi release in 15 min (µg) | Protein concn. (mg/ml) | Specific activity |
|---|---|---|---|---|---|
| **Adsorption Process** | | | | | |
| Crude Ext. 1 | 0 | 8.5 | 13.5 | 20 | 0.68 |
| Al supernat 1 | 1 | | 13.5 | | |
| Al supernat 2 | 2 | | 10.5 | | |
| Al supernat 3 | 2 | | 8.0 | | |
| Al supernat 4 | 2 | | 3.5 | 4.8 | 0.73 |
| **Elution Process** | | | | | |
| **Eluting agent** | **Elution time (hrs)** | | | | |
| 1 Water | 0.5 | 3 | 2.5 | | |
| 2 0.2M $NaHCO_3$ | 0.5 | 3 | 2.0 | 2.6 | 0.77 |
| 3 " | 0.5 | 3 | 2.5 | 3.05 | 0.80 |
| 4 0.5M $(NH_4)_2SO_4$ | 0.5 | 3 | 5.0 | 8.9 | 0.55 |
| 5 1.0M $NaHCO_3$ | 18 | 3 | 4.5 | 5.5 | 0.82 |
| 6 " | 0.5 | 2 | 8.0 | 4.6 | 1.70 |
| 7 " | 0.5 | 3 | 4.5 | 2.2 | 2.04 |
| 8 " | 2.0 | 3 | 6.0 | 2.0 | 3.0 |
| 9 " | 18 | 3 | 8.5 | 2.75 | 3.1 |
| 10 " | 2.0 | 3 | 4.0 | 1.45 | 2.8 |
| 11 " | 0.5 | 3 | 0.5 | | |
| 12 " | 18 | 3 | 3.0 | 1.45 | 2.1 |
| 13 0.1M Na citrate | 3.0 | 3 | 2.5 | 0.75 | 3.3 |
| 5-10combined | | 17 | 6.5 | 3.4 | 1.9 |

Table 12.

Fractionation of E. coli Ext. I on alumina Cγ (Expt. 29b).

| Protein Fraction | Vol. gel (ml) | Vol. Protein soln. (ml) | Pirelease in 15 min (µg) | Protein concn. (mg/ml) | Specific activity |
|---|---|---|---|---|---|
| **Adsorption Process** | | | | | |
| Crude Ext. 1 | 0 | 10 | 12.5 | 20.5 | 0.59 |
| Al supernat 1 | 2 | | 12.5 | 17.5 | 0.69 |
| Al supernat 2 | 8 | | 2.5 | 2.5 | 1.0 |
| **Elution Process** | | | | | |
| Eluting agent | Elution time (hrs) | | | | |
| 1 0.25M $(NH_4)_2SO_4$ | 1.0 | 4 | 3.0 | 4.9 | 0.77 |
| 2 " | 0.5 | 4 | 1.0 | 3.4 | 0.29 |
| 3 0.5M $(NH_4)_2SO_4$ | 0.5 | 4 | 4.5 | 3.8 | 1.18 |
| 4 0.1M Na citrate | 18 | 4 | 8.0 | 6.3 | 1.27 |
| 5 " | 0.5 | 4 | 6.5 | 3.4 | 1.91 |
| 6 " | 3.0 | 4 | 4.5 | 2.6 | 1.73 |
| 7 " | 1.0 | 4 | 3.5 | 2.0 | 1.75 |
| 8 " | 0.25 | 4 | 3.5 | 1.4 | 2.50 |
| 9 " | 18 | 4 | 2.5 | 1.8 | 1.39 |
| 10 0.2M Na citrate | 1.0 | 4 | 2.5 | 2.6 | 0.96 |
| 4-10combined | | 28 | 4.0 | 2.15 | 1.86 |

Table 13.

Fractionation of E. coli Ext. I on alumina Cγ (Expt. 29c).

| Protein Fraction | Vol. gel (ml) | Vol. Protein soln. (ml) | Pi release in 15min (µg) | Protein concn. (mg/ml) | Specific activity |
|---|---|---|---|---|---|
| **Adsorption Process** | | | | | |
| Crude Ext. 1 | 0 | 35 | 13.5 | 20.5 | 0.68 |
| Al supernat 1 | 9 | | 14.0 | 17.5 | 0.80 |
| Al supernat 2 | 30 | | 2.5 | 2.5 | 1.0 |
| Al supernat 3 | 10 | | 1.0 | 1.0 | 1.0 |
| **Elution Process** | | | | | |
| **Eluting agent** | **Elution time (hrs)** | | | | |
| 1 0.25M $(NH_4)_2SO_4$ | 2.5 | 30 | 1.0 | 2.1 | 0.48 |
| 2 0.5M " | 0.5 | 30 | 2.0 | 2.9 | 0.69 |
| 3 " | 18 | 15 | 2.0 | 2.9 | 0.69 |
| 4 1.0M $NaHCO_3$ | 1.0 | 30 | 2.5 | 3.8 | 0.65 |
| 5 " | 18 | 30 | 6.0 | 2.9 | 2.07 |
| 6 " | 2.0 | 30 | 9.0 | 2.6 | 3.45 |
| 7 " | 18 | 30 | 7.5 | 1.2 | 6.25 |
| 8 " | 3.0 | 30 | 4.5 | 0.75 | 6.0 |
| 9 " | 18 | 30 | 5.0 | 0.60 | 8.3 |
| 10 " | 24 | 30 | 5.0 | 1.2 | 6.4 |
| 11 " | 24 | 30 | 3.5 | .80 | 4.4 |

Table 14.

Fractionation of E. coli Ext. I on alumina Cγ (Expt. 29d).

| Protein Fraction | Vol. gel (ml) | Vol. Protein soln. (ml) | Pi release (µg) 5min | Pi release (µg) 15min | Protein concn. (mg/ml) | Specific activity |
|---|---|---|---|---|---|---|
| **Adsorption Process** | | | | | | |
| Crude Ext.I | 0 | 100 | 13.5 | | 19.4 | 2.25 |
| Al supernat 1 | 30 | | 12.5 | | 12.7 | 3.2 |
| Al supernat 2 | 120 | | | 8.5 | 1.9 | 5.1 |
| **Elution Process** | | | | | | |
| **Eluting agent** | **Elution time (hrs)** | | | | | |
| 1 0.25M $(NH_4)_2SO_4$ | 1 | 30 | | 7.5 | 1.15 | 7.7 |
| 2 " " $(NH_4)_2SO_4$ | 18 | 30 | | 6.0 | 0.82 | 7.7 |
| 3 0.5M $(NH_4)_2SO_4$ | 6 | 30 | | 9.0 | 0.87 | 9.2 |
| 4 " " $(NH_4)_2SO_4$ | 18 | 30 | | 7.5 | 0.67 | 11.1 |
| 5 1M $NaHCO_3$ | 1 | 30 | | 11.0 | 1.37 | 8.6 |
| 6 " | 18 | 30 | | 11.5 | 1.46 | 8.5 |
| 7 " | 1 | 30 | | 9.0 | 0.67 | 14.4 |
| 8 " | 18 | 30 | | 9.0 | 0.40 | 18.8 |
| 9 " | 1 | 30 | | 9.5 | 0.35 | 24.6 |
| 10 " | 1.5 | 30 | | 10.0 | 0.31 | 34.5 |
| 11 " | 1 | 30 | | 6.0 | 0.15 | 43.0 |
| 12 " | 18 | 30 | | 5.5 | 0.27 | 21.0 |
| 13 " | 48 | 30 | | 7.5 | 0.30 | 26.9 |
| 14 " | 18 | 30 | | 4.5 | 0.17 | 28.4 |
| 9-14 combined | | 180 | | 8.5 | 0.22 | 42.0 |

In some of the best fractions the activity is increased by a factor of ten over the crude extract. The recovery of polynucleotide phosphorylase is high, in some cases over 100%. This suggests that the crude extract contains an enzyme inhibitor which is removed during the purification. Solutions of the enzyme in 1M $NaHCO_3$ can be stored for several weeks at $4^{o}C$ without loss of activity.

Expt. 29d deserves some comment at this stage, since there is no doubt that a considerable part of the activity, particularly of later fractions low in total protein, is accounted for by the action of inorganic pyrophosphate on the contaminated ADP used in assaying this preparation. The presence of inorganic pyrophosphate in this ADP sample had not been recognised at the time the experiment was carried out and those fractions of apparently high specific activity were combined for further experiments. Addition of later, dilute fractions would cause some dilution of the polynucleotide phosphorylase in earlier fractions. The first eluted fractions (1-8) which appeared to be of lower specific activity but which, in the light of later experience, were probably the most active, were discarded.

The enzyme preparation resulting from a combination of fractions 9-14 of Expt. 29d was used in many, later experiments in which attempts were made to purify the enzyme further. It will be seen, that some of the methods applied for this purpose, resulted in enzyme preparations of genuinely high polynucleotide phosphorylase activity.

The experience of Expts. 29 a-d showed that in order to obtain solutions of high protein concentration, extended elution periods were necessary. In addition, each elution must be followed by centrifugation and assay of the supernatant solution. As treatment of a gel sample required over 12 separate elution steps, the process required a week of constant manipulations.

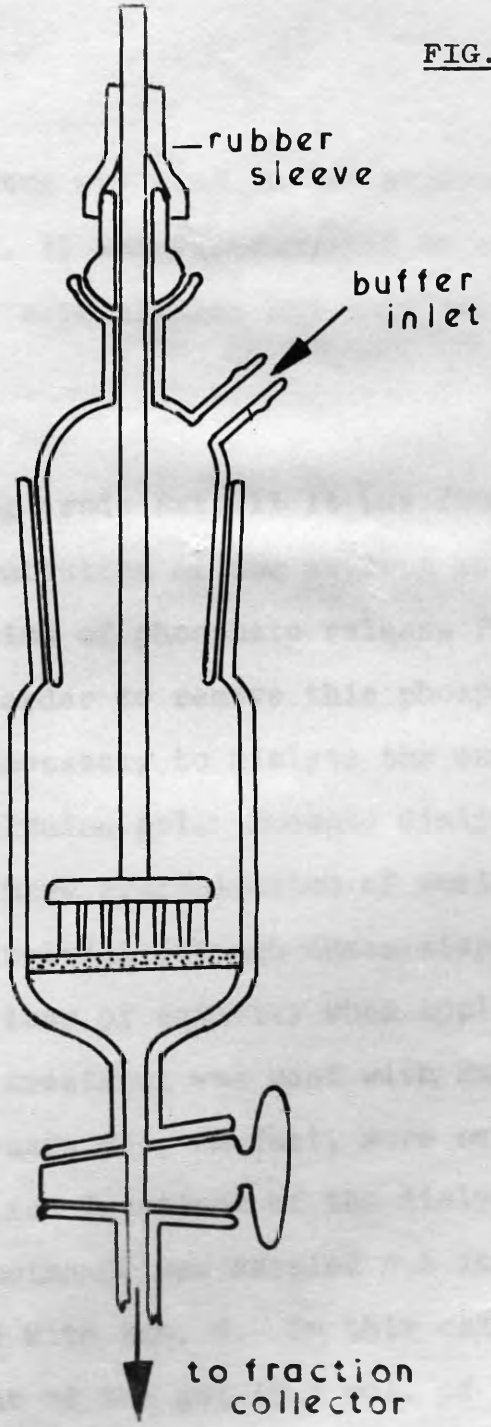
In order to simplify the elution procedure, a device was developed which made it possible to elute the gel continuously and which also permitted use of an automatic fraction collector. The gel is contained in a vessel which is divided by a sintered glass filter (porosity 3, 1¼"dia.). Beneath the sinter the vessel narrows and enters a glass stopcock. The upper part of

the vessel is a B34 ground-glass socket into which a head, bearing a ball and socket stirrer and an inlet arm, can be fitted. The apparatus is illustrated in Fig. 19. The gel is washed onto the sinter and eluted by the solution which enters the inlet tube. Its flow-rate is controlled by adjusting the outlet tap (for decreased flow) or the height of the reservoir (for increased flow). Initially, stirring was effected by a glass spiral stirrer, but it was found that the high speeds, required to keep the gel in suspension and to prevent it from clogging the sinter, caused frothing. This difficulty was overcome by using a stirrer constructed from the head of a nylon toothbrush. This was fixed by a stainless steel screw to a rod of the same material. This stirrer enabled the surface of the sinter to be kept clear even with slow stirring.

In addition to the advantages provided by continuous operation and automatic collection of fractions, it is possible, using this apparatus, to apply the eluting agent in continuously increasing concentration (gradient elution) rather than in the step-wise increments which are applied in the more usual

Apparatus for the continuous elution of gels

FIG. 19



method of elution.

The apparatus was used in two experiments in which protein of Ext. II was fractionated on alumina C$\gamma$ . The results of these were similar and only one of them is described here.

On assaying crude Ext. II it was found that the orthophosphate concentration of the extract was so high that an estimation of phosphate release from ADP was impossible. In order to remove this phosphate from the extract it was necessary to dialyse the extract before adding the alumina gel. Because dialysis was necessary, a preliminary precipitation of nucleic acids with manganese was included (though these steps apparently caused considerable loss of activity when applied to Ext. I). When this treatment was used with Ext. II (Expt. 30) the dialysate was, in fact, more active than untreated crude Ext.I. Treatment of the dialysed manganese supernatant with alumina C$\gamma$ was carried out in a manner similar to that used with Ext. I. In this case, however, a much smaller amount of the gel (0.2 vol. of the protein

solution vs. 1.4 vol. for crude Ext. I) was required to adsorb the active protein.

The course of the adsorption is illustrated in Fig. 20 (see also Table 15).

The last two fractions of gel (4 and 5) contained 60% of the polynucleotide phosphorylase activity and 45% of the protein. These were washed into the elution apparatus, described above, and eluted with saturated aqueous sodium bicarbonate solution. Elution was carried out very slowly (approx. 1.5 fractions per day) and the fractions, which were all deep yellow in colour, were assayed for protein and polynucleotide phosphorylase activity. It was these assays which gave the first indications that the E. coli enzyme could demonstrate a lag phase. The first set of assays were carried out using a 60 min. digest period instead of the customary 15 min. in order to produce a higher phosphate release and thus increase the sensitivity of the assay. The results were unexpectedly high (see Table 15) and the assays were repeated using a 15 min.

FIG. 20

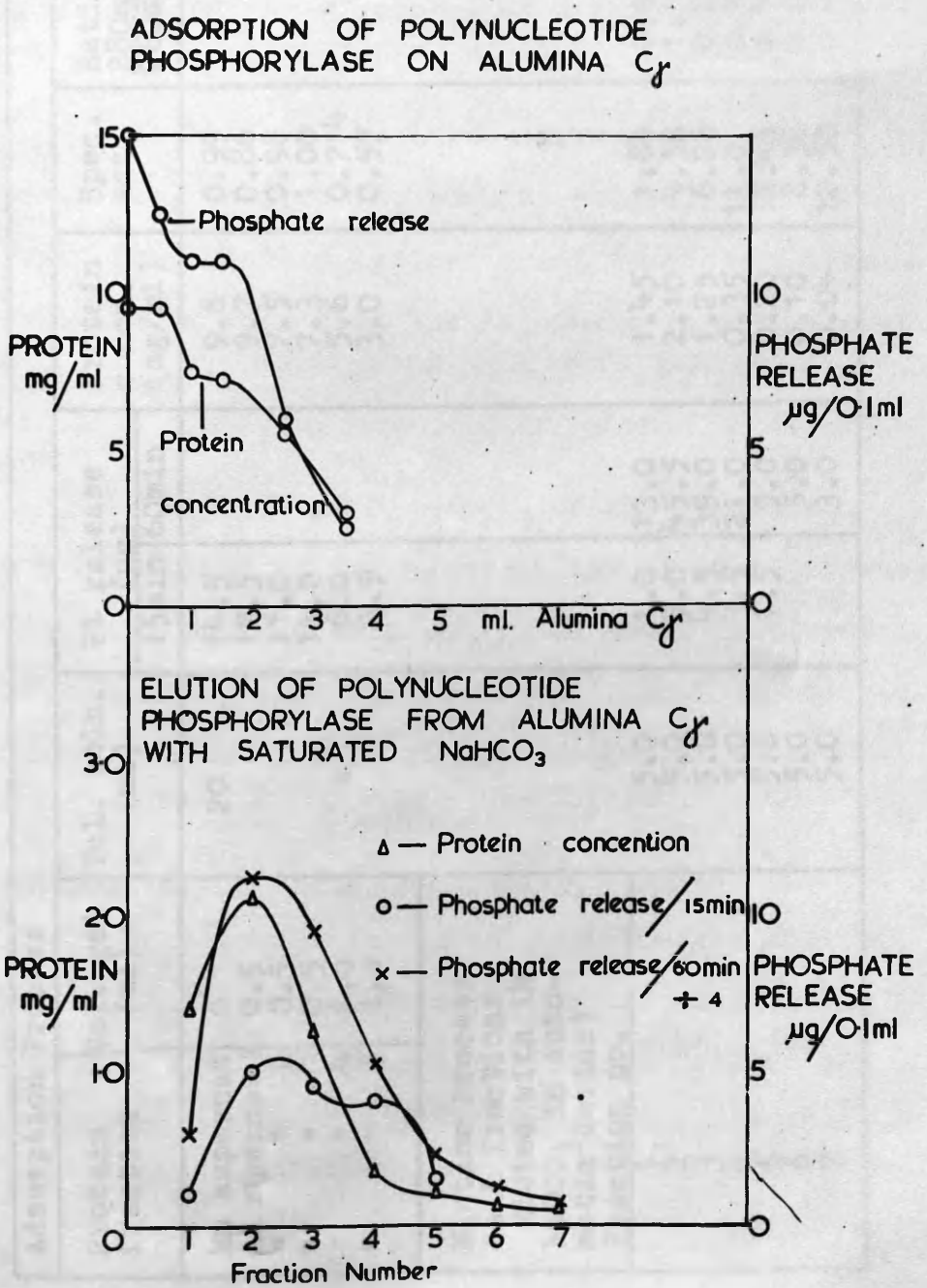

ADSORPTION OF POLYNUCLEOTIDE PHOSPHORYLASE ON ALUMINA Cγ
15
10
5
0
Phosphate release
Protein concentration
PROTEIN mg/ml
PHOSPHATE RELEASE µg/0·1ml
1 2 3 4 5 ml. Alumina Cγ
ELUTION OF POLYNUCLEOTIDE PHOSPHORYLASE FROM ALUMINA Cγ WITH SATURATED NaHCO3
3·0
2·0
1·0
Δ — Protein concention
o — Phosphate release/15min
× — Phosphate release/60min + 4
PROTEIN mg/ml
PHOSPHATE RELEASE µg/0·1ml
1 2 3 4 5 6 7
Fraction Number

Table 15.

Fractionation of E. coli Ext. II (manganese treated) on alumina Cγ (Expt. 30).

| Adsorption Process | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Protein fraction | Vol. gel (ml) | Vol. soln. (ml) | Pi release (µg) 15min | Pi release (µg) 60min | Protein concn. (mg/ml) | Spec. act. | Ratio 280mµ / 260mµ | % Nucleic acid |
| Mn supernat | 0 | 20 | 14.5 | | 9.8 | 0.99 | | |
| Al supernat 1 | 0.5 | | 12.5 | | 9.7 | 0.86 | | |
| " " 2 | 0.5 | | 11.0 | | 7.5 | 0.98 | | |
| " " 3 | 0.5 | | 11.0 | | 7.3 | 1.00 | | |
| " " 4 | 1.0 | | 6.0 | | 5.6 | 0.714 | | |
| " " 5 | 1.0 | | 2.5 | | 3.0 | 0.57 | | |
| Elution Process (all fractions eluted with 1M $NaHCO_3$ in automatic device) Fraction no. | | | | | | | | |
| 1 | | 5.0 | 1.0 | 13.0 | 1.45 | 1.84 | 0.93 | 4 |
| 2 | | 5.0 | 5.0 | 45.5 | 2.10 | 4.38 | 1.09 | 2.5 |
| 3 | | 5.0 | 4.5 | 39.0 | 1.25 | 6.28 | 0.83 | 6 |
| 4 | | 5.0 | 3.5 | 21.0 | 0.35 | 11.0 | 0.73 | 9 |
| 5 | | 5.0 | 1.5 | 9.0 | 0.20 | 8.43 | 0.71 | 10 |
| 6 | | 5.0 | | 5.0 | 0.10 | 8.30 | 0.66 | 12 |
| 7 | | 5.0 | | 3.0 | 0.04 | 12.50 | 0.44 | 20 |

digest period. These showed (Table 15, column 4) that the phosphate release over 15 min. is much less than a quarter of that released in 60 min. This can only be explained by the existence of a lag phase.

In Fig. 20 the three curves correspond, respectively, to protein, 60 min. activity and 15 min. activity (x4) plotted against fraction number. From this it can be seen that the lag-phase is most pronounced with fraction 1.

The ratio of $D_{280}/D_{260}$ of the fractions (Table 15, column 9) decreases as the elution progresses, showing that nucleic acid is eluted later than protein. It is noticeable that the fractions lowest in nucleic acid concentration show the most pronounced lag phase.

Further purification of the alumina eluate.

Despite the degree of purification achieved by the fractionation on alumina Cγ, the preparation was not satisfactory for the preparation of polyU. An early enzyme sample (Table 11, Expt. 29a) was shown

to contain sufficient RNase to release 6 micrograms of acid soluble phosphorus from RNA in ½ hr., under the conditions normally employed for polynucleotide phosphorylase assay. The same enzyme preparation released 15 micrograms of inorganic phosphorus during the synthesis of polyA from ADP. Thus the concentration of RNase, though low, would cause serious degradation of polyU synthesised by this sample of polynucleotide phosphorylase. Attempts were therefore made to purify the enzyme further, and particular attention was paid to removing the RNase. Most of this work was carried out before the discovery of the contaminant in the commercial ADP which had been used to assay Expt. 29d, the large scale fractionation on alumina Cγ. The specific activity of this sample is therefore unknown and it is unfortunate that this was used in the search for methods to purify this fraction further. However, the sample does contain polynucleotide phosphorylase and in view of the procedure used in its preparation, the results obtained in subsequent treatment should apply to other similar alumina eluates.

It should be borne in mind that at the time this work was carried out, the existence of the lag phase

was not discovered. This is more likely to interfere with the assay method the further the enzyme is purified from nucleic acid. It is possible ,therefore,that many fractions of high purity have been discarded because over a 15 or 30 min. assay period they showed no activity.

Removal of RNase from alumina Cγ eluate by bentonite treatment

The use of bentonite as an adsorbent for ribonuclease has been described previously. Its success with crude E. coli extracts indicated that it might also be useful with the partially purified enzyme. The small amount of RNase present in the alumina Cγ eluate is, in fact, readily removed by adding bentonite. This step can be carried out on the undialysed alumina eluate; indeed the sodium bicarbonate present in the eluate decreases the adsorption of polynucleotide phosphorylase. A preliminary experiment readily shows the proportion of bentonite required to adsorb all of the RNase without concomitant removal of polynucleotide phosphorylase.

Three different samples of the alumina eluates have been treated in this way. A typical procedure is describe in Expt. 31. The effect on polynucleotide phosphorylase and protein concentration of adding increasing amounts

of bentonite to alumina eluate from Expt. 30 is shown in Table 16. Ribonuclease was not detectable after the first addition of the adsorbent.

The enzyme prepared by fractionation on alumina C$\gamma$ and treatment with bentonite is suitable for the synthesis of polyU from UDP and two samples were used, in large scale experiments for the preparation of polyU, described later. These were derived from alumina eluates 29d and 30.

PolyA synthesis using polynucleotide phosphorylase is limited by the presence in the enzyme preparation of a high concentration of adenylate kinase which reduces the ADP concentration. A contaminant which is less desirable in the present work is nucleic acid. In the active fractions of alumina eluate (Expt. 30), the concentration of nucleic acid is 6% of the total protein, even though in this preparation, the nucleic acids were precipitated with manganese before alumina fractionation. It will be seen later that the presence of this material is the most likely cause of the failure to hydrogenate one

Table 16

Treatment of alumina C/eluate with bentonite.

| Volume of bentonite | Phophate release ( ug/15 min) | Protein concn. (mg/ml.) |
|---|---|---|
| 0 | 12.5 | 15.5 |
| 0.25 | 11.0 | 10.5 |
| 0.05 | 8.0 | 8.0 |
| 0.75 | 3.5 | 7.5 |
| 0.1 | 2.5 | 8.0 |

sample of polyU.

Other methods of purification applied to the alumina eluate.

Other fractionation methods were applied to the alumina eluate and although these were not used in a large scale preparation of the enzyme for the purpose of synthesising polyU, they are reported here, briefly. The enzyme is known to be acidic, from its behaviour with bentonite and from its electrophoretic mobility (179). Therefore, chromatography on the anion exchange cellulose DEAE appeared to offer a possible approach to purification. A preliminary experiment (Expt. 32) showed that the activity is readily absorbed from the dialysed alumina eluate (from Expt. 29b) by passing through a small column of DEAE ($Cl^-$). Subsequent elution of the column with solutions of potassium chloride of increasing concentration, removes the activity (at 0.2M KCl).

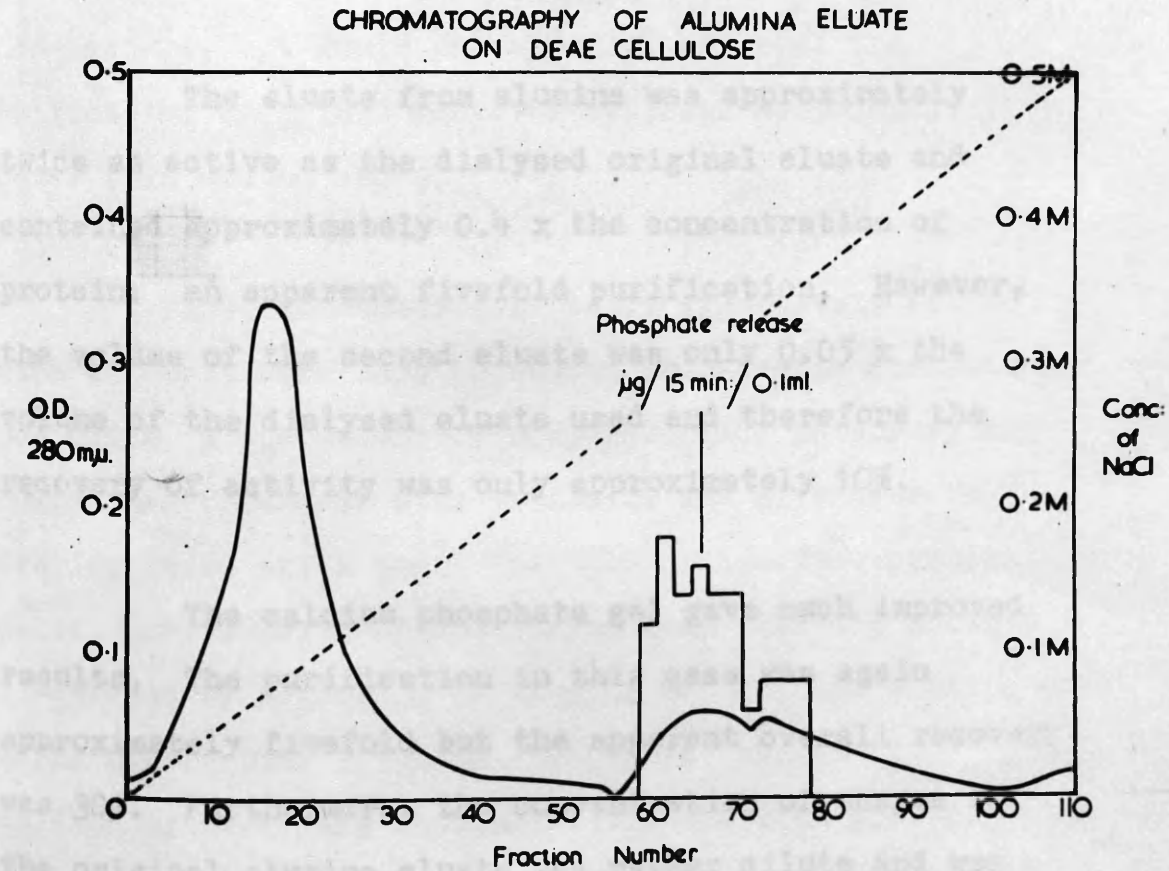
In a more refined experiment (Expt. 33) the dialysed eluate was applied to a larger column of DEAE and this was eluted by applying a linearly increasing

gradient of NaCl (0.01M to 0.5M). Fractions were collected automatically and assayed for activity and protein concentration. The elution pattern is shown in Fig. 21.

The initial, large protein peak eluted between fractions 10 and 27 was apparently inactive. A second and very dilute protein peak did contain phosphate releasing activity. However, the phosphate release from each fraction in this peak is approximately the same, irrespective of protein concentration and this suggests that the peak contains inorganic pyrophosphatase activity rather than polynucleotide phosphorylase. Although this was not realised at the time, and the activity was thought to be due to polynucleotide phosphorylase, the dilution of the protein resulting from the fractionation did not encourage further development of the method.

Better results were obtained by using the adsorbents alumina Cγ and calcium phosphate. Each of these was able to remove most of the activity when a

FIG. 21


CHROMATOGRAPHY OF ALUMINA ELUATE
ON DEAE CELLULOSE
0·5
0·4
0·3
0·2
0·1
O.D.
280mμ.
0·5M
0·4M
0·3M
0·2M
0·1M
Conc: of NaCl
Phosphate release
μg/15 min:/0·1ml.
0
10
20
30
40
50
60
70
80
90
100
110
Fraction Number

small amount was added to the dialysed alumina eluate (Expts. 34,35). Again the aluminaC3 was the more active of the adsorbents. Elution of the gels with saturated sodium bicarbonate led to the following results.

The eluate from alumina was approximately twice as active as the dialysed original eluate and contained approximately 0.4 x the concentration of protein: an apparent fivefold purification. However, the volume of the second eluate was only 0.05 x the volume of the dialysed eluate used and therefore the recovery of activity was only approximately 10%.

The calcium phosphate gel gave much improved results. The purification in this case was again approximately fivefold but the apparent overall recovery was 30%. Furthermore, the concentration of enzyme in the original alumina eluate was rather dilute and was concentrated threefold by the calcium phosphate treatment.

As was pointed out previously, these experiments were carried out on the alumina eluate from Expt. 29d

which had been assayed using the contaminated ADP and this was also used in assaying the subsequent purification methods described above. If the error due to phosphate release from inorganic pyrophosphate could be taken into account, it would undoubtedly show that the purification and yield achieved by these methods is better than stated above.

Thus, to summarise: early experiments in which attempts were made to remove undesirable contaminants from crude preparations of polynucleotide phosphorylase showed that treatment with bentonite was effective in removing RNase and a small fraction of inactive protein. Partial purification of the enzyme from inactive protein is achieved by adsorbing on alumina $C\delta$ gel and eluting with sodium bicarbonate solution. Improved results are realised by preceding alumina adsorption with precipitation of nucleic acids as the manganese salts and subsequently dialysing the extract. Elution of the gel is most conveniently carried out using the apparatus designed for this purpose. Small amounts of RNase in the eluate from alumina $C\delta$ are readily adsorbed on bentonite.

Further purification of the alumina eluate by fractionation on calcium phosphate gel gave most promising results. However, this and less successful attempts using alumina $C^{\alpha}$ and DEAE ion exchange cellulose have not been repeated on protein preparations of known activity and using a reliable assay method, because the simpler bentonite method appeared to give an enzyme preparation suitable for the present purpose.

Synthetic pyrophosphate esters as substrates for polynucleotide phosphorylase.

Ribose-5-pyrophosphate.

It was hoped that a ribose phosphate polymer would result from the action of polynucleotide phosphorylase on ribofuranose-5-pyrophosphate. The pyrophosphate ester (lithium salt synthesised in Expt. 15) was incubated with enzyme (Expt. 36). As judged by phosphate release no reaction took place, even after 2 days incubation.

4:5-Dihydrouracilriboside-5'-pyrophosphate.

The action of polynucleotide phosphorylase on 4:5-dihydrouracilriboside-5'-pyrophosphate

was studied with the expectation that polydihydrouridylic acid might be prepared directly in this way. The ester prepared in experiment 17 was treated with enzyme (Expt. 37). Even prolonged digestion did not lead to release of inorganic phosphate. Since this work was carried out a similar result has been reported by other workers(189).

Uridine-5'-pyrophosphate.

The synthesis of polyuridylic acid from UDP is described in Expts. 38 and 39. In the first, smaller experiment, enzyme prepared in Expt. 29d and subsequently treated with bentonite, was used.

The course of the reaction was followed by withdrawing aliquots for inorganic phosphate estimation (Fig. 22). According to the amount of phosphate released at the end of reaction, the yield of polymer was approximately 30%. The mixture after reaction had ceased, was fractionated on a column of Sephadex G 25 (this method is discussed at the end of this section and in G.M. 18). Two peaks corresponding,

respectively, to polyU and monomeric material were eluted. According to the total optical density at 260mμ of the two peaks, the yield of polymer was approximately 20%.

In the large scale preparation of polyU, the enzyme used was bentonite treated alumina eluate from Expt. 30. The UDP was used as the lithium salt, prepared in Expt. 16.

The fractionation of the reaction mixture on Sephadex G25 is shown in Fig. 23b. After isolation from the column, polyU and UDP were recovered by freeze-drying. The yield by weight was 25% of theory. Deproteinisation of the polyU solution was effected by a treatment with bentonite.

Chemical synthesis of short-chain polyU.

The method of Michelson (190) was used to prepare polymers of polyU for model experiments described in Section III. The polymers prepared in this way are of intermediate chain length and are joined through both 2'-5'- and 3'-5'- phosphodiester linkages in random sequence. Because of this, the polymers prepared in this way were not

FIG. 22

Synthesis of polyuridylic acid by

polynucleotide phosphorylase

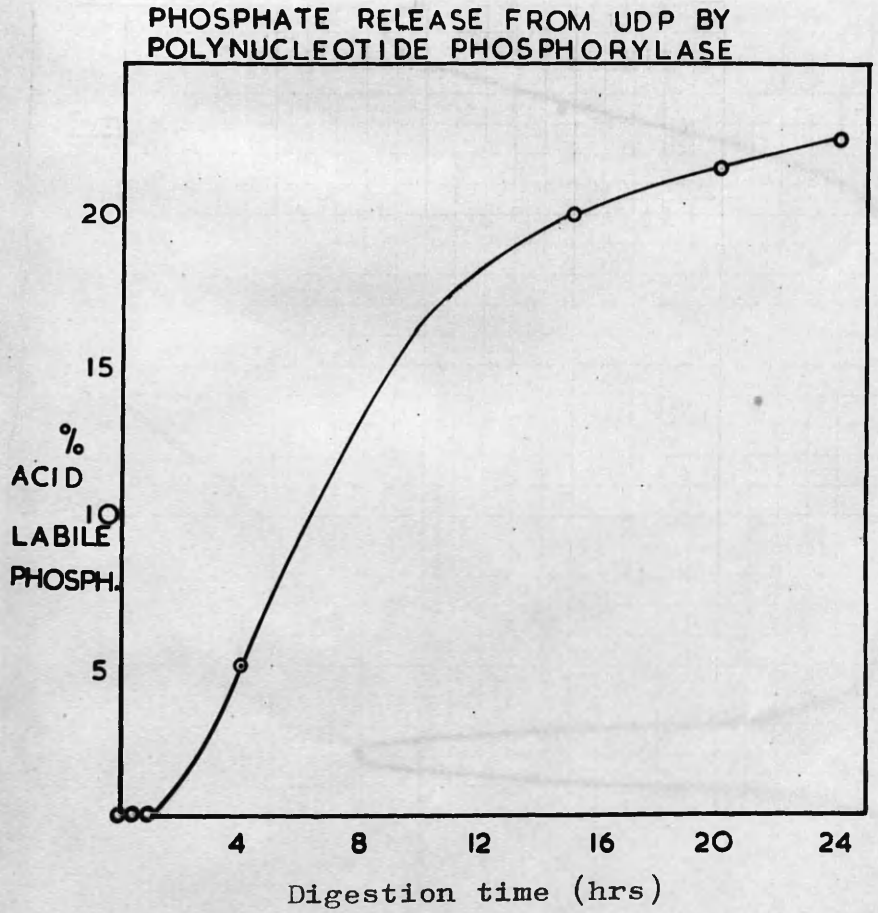


FIG. 23a

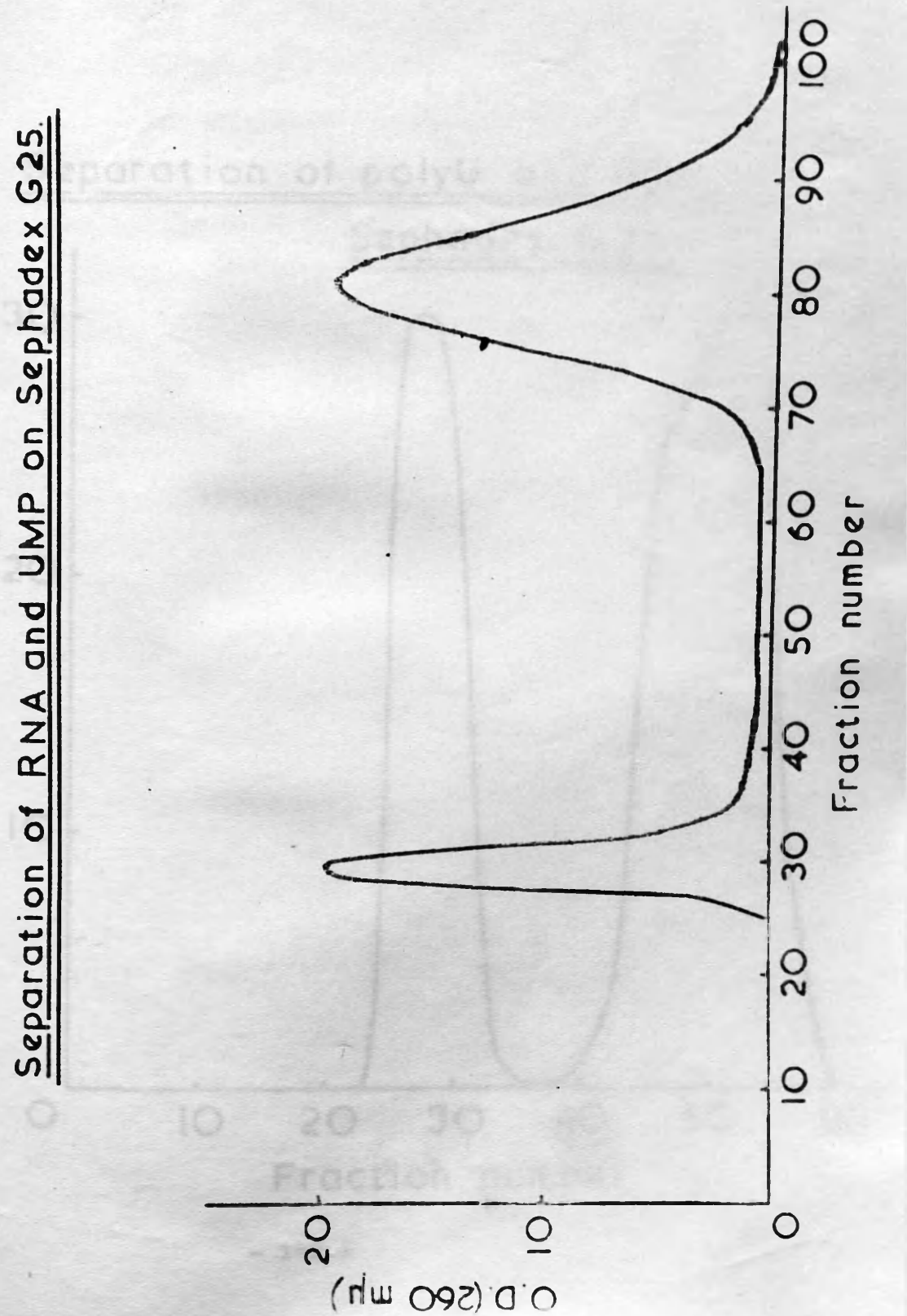
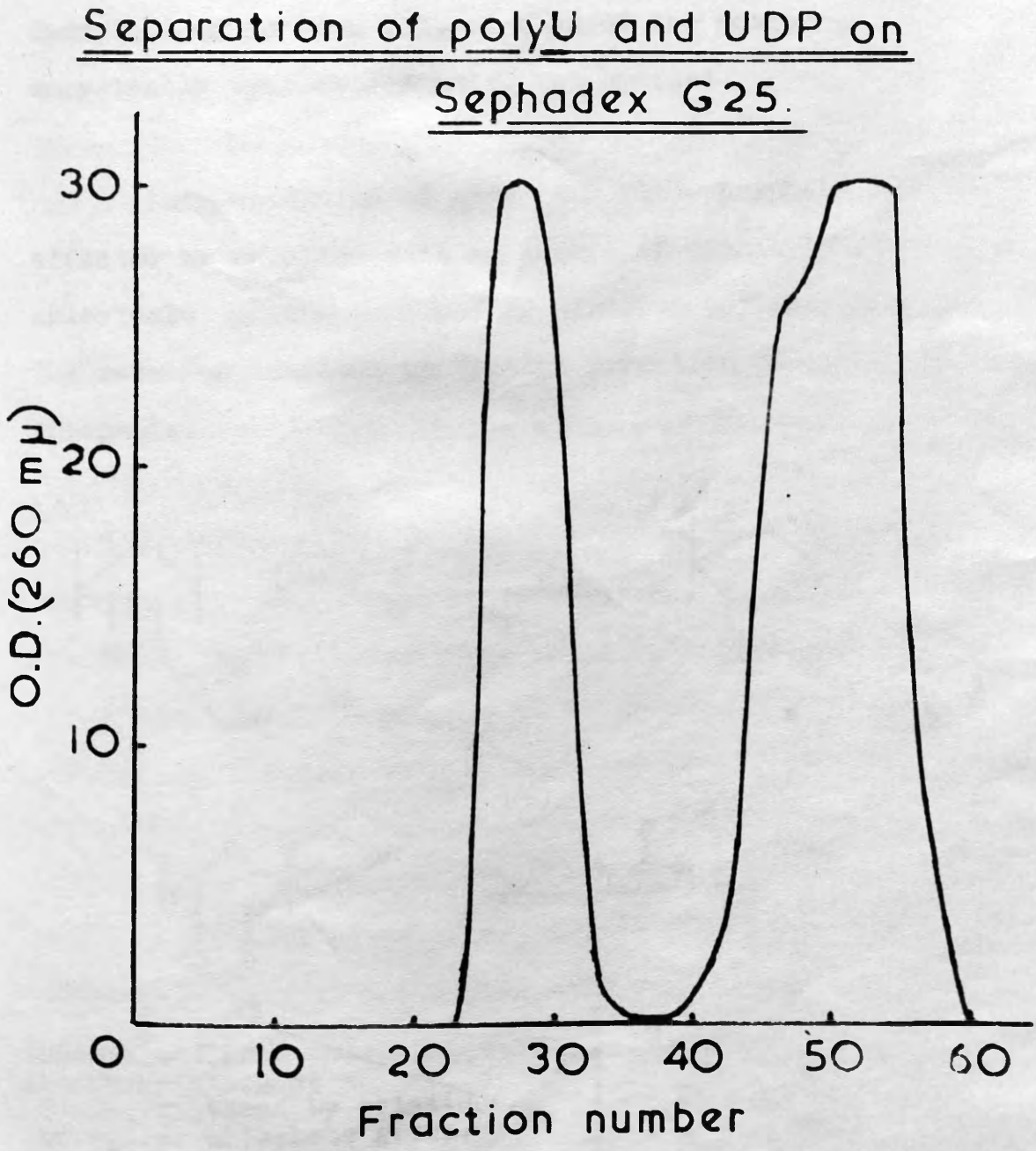
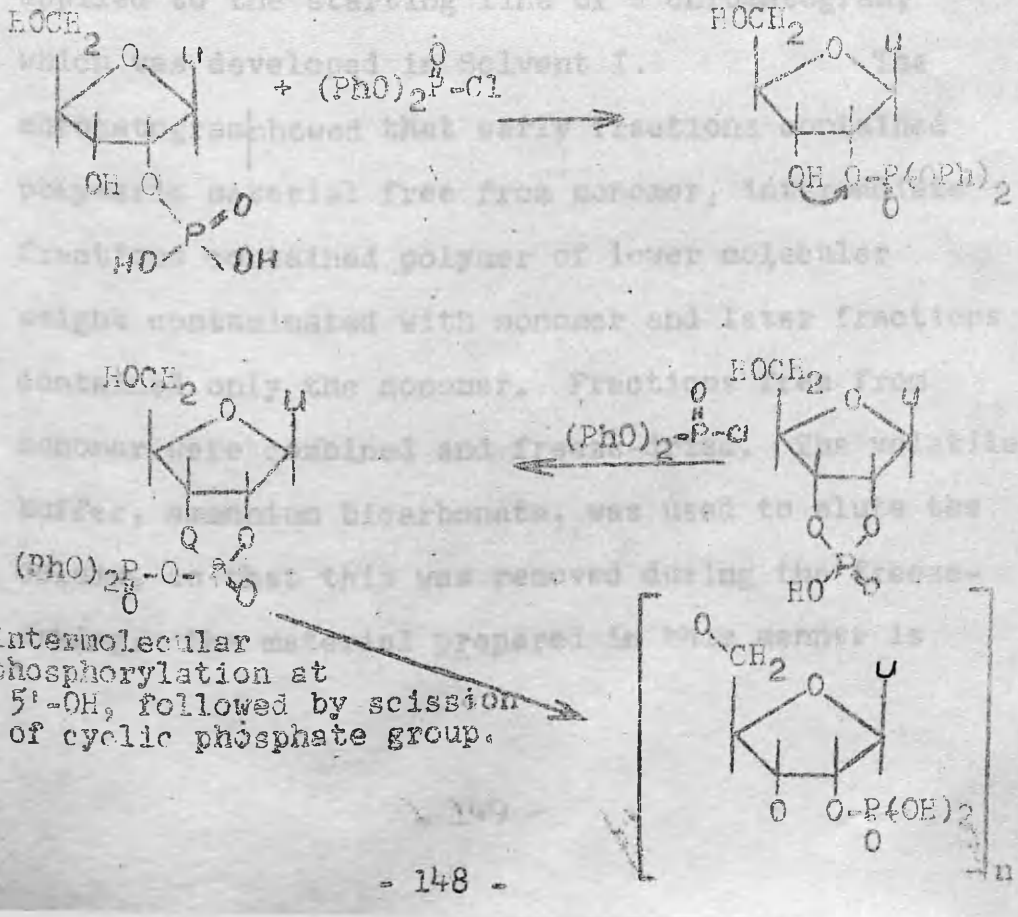

Separation of RNA and UMP on Sephadex G25.
O.D (260 mμ)
0
10
20
Fraction number
10
20
30
40
50
60
70
80
90
100

FIG. 23b


Separation of polyU and UDP on Sephadex G25.
O.D.(260 mμ)
30
20
10
0
10
20
30
40
50
60
Fraction number

considered suitable for the preparation of a ribose phosphate polymer for the enzymic studies. However, by using these materials in the exploratory studies described in Section III, wastage of the scarcer, enzymically synthesised polyU was avoided.

Polymerisation of uridine-2'(3')-phosphate is effected by reaction with an excess of diphenylphosphorochloridate, in the presence of hindered tertiary base. The reaction involves an initial formation of cyclic phosphate:



The polymerisation reaction was carried out exactly as described by Michelson, (Expt. 40) but after removal of organic solvents and excess reagent, the product was taken up in water and fractionated on Sephadex G 25. This avoids the magnesium salt fractionation employed by Michelson, which, in a preliminary trial was found to give low yields.

The optical density at 260 mμ of the fractions eluted from Sephadex was determined, and aliquots of those containing appreciable amounts of material were applied to the starting line of a chromatogram, which was developed in Solvent I. The chromatogram showed that early fractions contained polymeric material free from monomer, intermediate fractions contained polymer of lower molecular weight contaminated with monomer and later fractions contained only the monomer. Fractions free from monomer were combined and freeze-dried. The volatile buffer, ammonium bicarbonate, was used to elute the column, so that this was removed during the freeze-drying. The material prepared in this manner is

henceforward referred to as chem. synth. polyU (Sephadex fractionI).

A note concerning the separation of polynucleotides from monomeric material on Sephadex and ECTEOLA.

For the isolation of polyribonucleotides synthesised by polynucleotide phosphorylase, other workers have used precipitation with acid, ethanol and strychnine. Grunberg-Manago who has reviewed these methods has pointed out (102) that ethanol precipitation is the only method suitable for the isolation of polyU. A trial experiment with this method on the polymer synthesised in Expt. 38 showed that the recovery of the polymer was low. It was decided, therefore, that for this separation advantage might be taken of the molecular seive materials which had been recently introduced.

These materials, which are marketed under the trade name "Sephadex" (Pharmacia, Uppsala) consist of granular, cross-linked dextrans and are available in forms which differ in the degree of cross-linkage

and therefore in molecular sieving properties. The G25 grade, used in this work, is able to exclude any material whose molecular weight exceeds approximately 25,000. The preparation of columns of Sephadex G25 is described in G.M. 18. A separation of RNA and UMP on such a column is shown in Fig. 23a; Fig. 23b shows the separation of polyU and UDP on the same column and it is clear from the latter that UDP is partially excluded by the molecular sieve and could be separated from UMP by this method. This observation led to the use of Sephadex G25 for the fractionation of chem. synth. polyU described above.

The molecular sieving technique was excellent for the purpose of isolating the polymeric material on a preparative scale, particularly in the case of the enzymically synthesised polyU, where the molecular weight difference is sufficiently large to ensure a complete separation from low molecular weight material. In experiments to be described later, a technique was required for the routine separation on an analytical scale of chem. synth. polyU (Sephadex

fraction I) and related polymers from low molecular weight compounds. Molecular sieving as described above, is not ideal for such work because it is difficult to predict at exactly what volume each peak will appear and it is therefore necessary to collect and analyse a large number of fractions from each column, to ensure separation. However, it was used in some analytical scale experiments described in Section III.

An improved method has been developed, which lends itself well to the routine separation of polyribonucleotide of intermediate chain length from low molecular weight material. The new method (G.M. 19) is based on the use of the ion-exchange cellulose ECTEOLA. The mixture to be separated is applied in dilute solution to this material prepared according to G.M. 19 (usually a very small column is sufficient, e.g. 5mm x 5mm for 2 mg material). Then, by choosing eluants of suitable salt concentrations, it is possible to remove the low molecular weight material entirely in a single fraction and the high molecular

weight material entirely in another, thus avoiding the necessity to collect a large number of fractions from each column. The usefulness of the method is illustrated by the chromatographic profiles (Fig. 24) obtained for (a) U2'(3')P and (b) chem. synth. polyU (Sephadex fraction I). These show that the fraction of (a) eluted by the stronger salt solution and the fraction of (b) eluted by the weaker salt solution are insignificant.

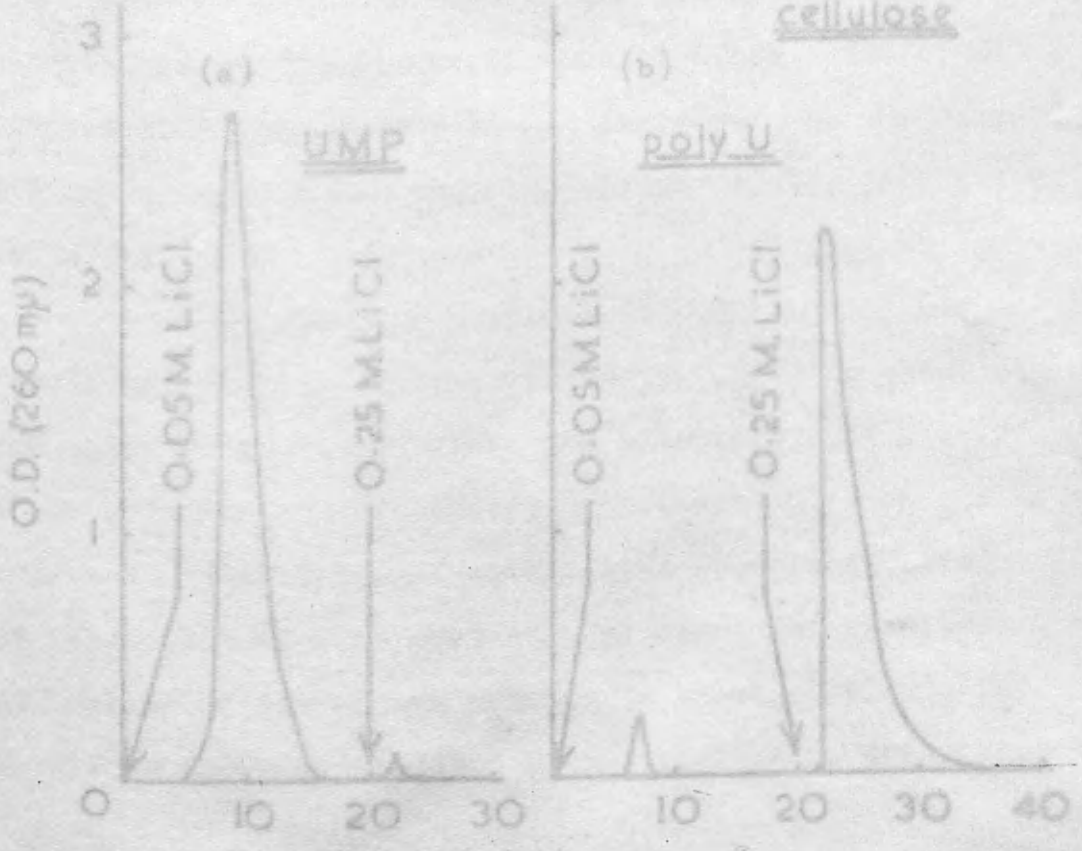
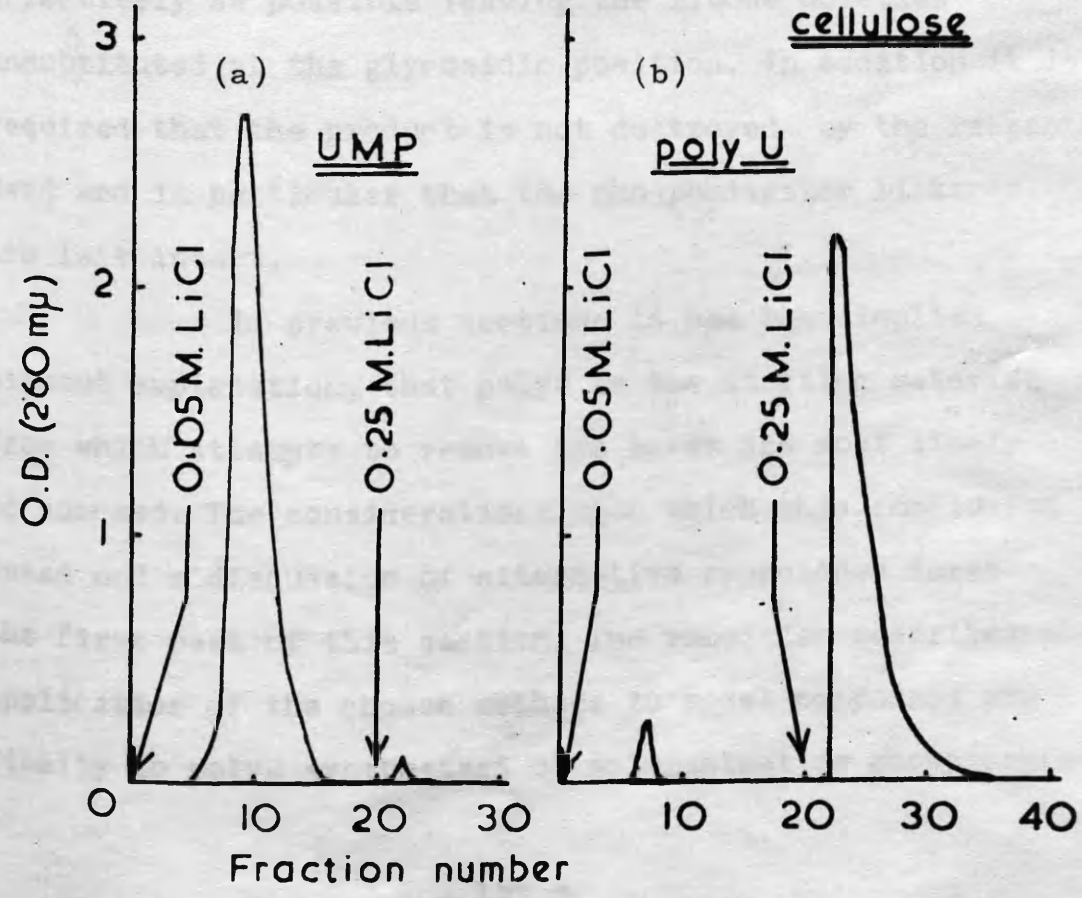


FIG. 24

Elution of UMP and chem. synth. poly U Sephadex fraction I from columns of ECTOLA-cellulose



## SECTION III.

### The Synthesis of a Ribose phosphate polymer from polyuridylic acid.

### Introduction.

The final stage of the present project, the removal of bases from an intact ribopolynucleotide, is described in this section. It is required of the method chosen to do this, that it shall remove the bases as effectively as possible leaving the ribose moieties unsubstituted at the glycosidic position. In addition it is required that the product is not destroyed by the reagents used and in particular that the phosphodiester linkages are left intact.

In previous sections it has been implied without explanation, that polyU is the starting material from which attempts to remove the bases are most likely to succeed. The considerations upon which this conclusion was based and a discussion of alternative approaches forms the first part of this section. The remainder describes the application of the chosen methods to model compounds and finally to polyU synthesised by polynucleotide phosphorylase.

The choice of a starting material.

Of the various glycosidic linkages which occur in nucleic acids, it is well known that the most labile to hydrolysis are those which involve a purine base. Thus, treatment of RNA with acid under mild conditions leads to a product known as apurinic acid, in which the purine bases have been removed (190), the phosphodiester chain remaining for the most part, intact. In contrast the pyrimidine ribosides are very stable to acid and will survive 12N perchloric acid for 1hr. at 100°C. (191).

It would appear, then, that removal of purine bases by acid hydrolysis from a ribopolynucleotide containing a single purine base might offer a promising route to a ribose phosphate polymer. Unfortunately, the internucleotidic linkage in RNA is much more labile to acid than the corresponding linkage in DNA. Unless the rate of hydrolysis of the glycosidic linkage is much higher than that of the internucleotidic linkage the product of acid hydrolysis will be at best a ribosephosphate polymer of very short chain length. Though no quantitative data concerning the rate of acid hydrolysis of polyribonucleotides is available Khym and Cohn have studied the acid hydrolysis of adenine nucleotides (192) and have shown that they are converted to the

corresponding ribose phosphates by treatment with an acidic exchange resin at 100°C in a few minutes. However under the same conditions, the rate of migration of the phosphate group from the 2'- to the 3'- position was high and this indicates that if this method were applied to a polyadenylate chain degradation would be extensive.

Mechanisms involved in the hydrolysis of N-ribotides are discussed in Appendix A.

The pyrimidine glycosidic linkage, as was mentioned previously, is an unusually stable one and this has been attributed to the 4,5-double bond in the pyrimidine ring. When this bond is removed by bromination (193) or hydrogenation (194) the acid lability becomes comparable to that of the purines. Even greater acid lability was achieved by the modifications achieved by Cohn and Doherty (195). This new method involved an initial reduction of the 4,5-double bond by hydrogenation over 5% rhodium on alumina as catalyst. Treatment of the dihydro derivative with mild alkali cleaves the dihydropyrimidine ring and renders the glycosidic linkage more labile to acid hydrolysis.

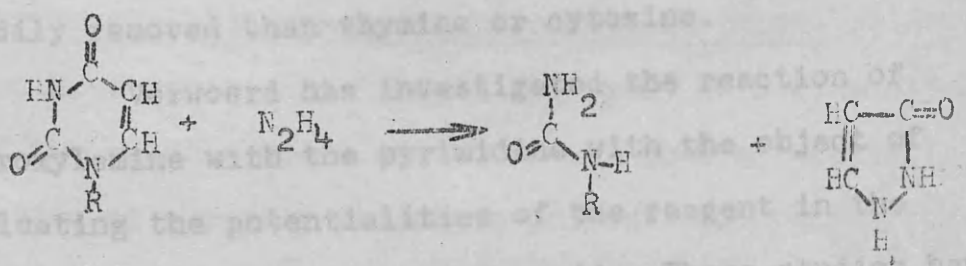
This series of reactions was used by Cohn and Doherty (195) in preparing the ribose phosphates from

the isomeric uridylic acids, which led to a proof of the structures of these nucleotides.

The conditions of hydrolysis are so mild that no migration of the phosphate group between the 2' and 3' positions takes place. It appears that this method(hencaforth referred to as Cohn's method) offers a promising route to a ribose phosphate polymer if it is applicable to polyU or polyC. In choosing between the two pyrimidines, polyU is favoured because of the greater ease with which uracil is reduced. (195). A related method has been applied to the deoxypyrimidine ribosides and ribotides by Laland et al (196) who showed that reduction of the base over sodium amalgam renders the glycosidic linkage exceptionally labile to hydrolysis by an acidic cation exchange resin.

Although the product of reduction is not clearly defined,this method appeared to offer possibilities in the present work. Preliminary experiments were carried out, in which U5'P was reduced over sodium amalgam under the conditions used by Laland et al for the reduction of the deoxyribotides. The products were analysed by paper chromatography in a manner similar to that described later for other treatments of U5'P studied in the present work. It was found that sodium amalgam reduction gave rise to at least four phosphate containing products from U5'P. This result discouraged further study of the method

Fosse et al(197 ) first reported the reaction between uracil and hydrazine and showed that the products are urea and pyrazolone. The reaction has since been applied to uridine (198) and uridylic acids (199). In each case the principle product; pyrazolone and a derivative of urea (N-ureide).

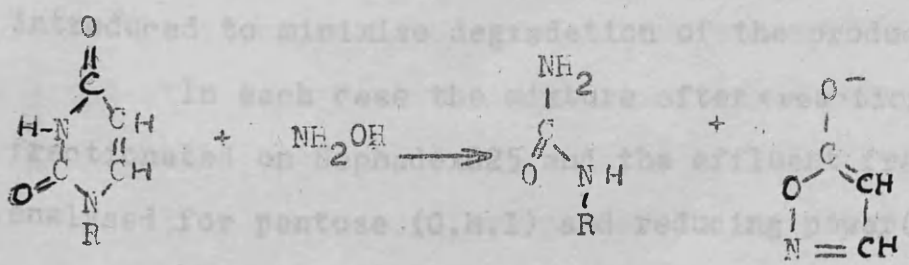


The process is referred to as hydrazinolysis. Baron and Brown subjected uridylic acids a and b to hydrazinolysis in hydrazine hydrate at 70°C and observed the formation of ribose 2-and3- phosphates respectively.These authors came to the conclusion that the N-ureide formed initially is very labile and is hydrolysed during isolation to the free ribose phosphate.The same conclusion was reached by Takamura( 200 ) who applied the reaction to intact nucleic acids: this point will be examined in greater detail later and dealt with in appendix A

The Japanese workers applied the technique to RNA, DNA, and apurinic acid, and named the products apyrimidinic acid, deoxyribopyrimidinic acid and polydeoxyribose phosphate respectively.

They found that although some chain degradation occurred during hydrazinolysis the products were still of intermediate molecular weight. The degree of removal of the pyrimidines depended upon the conditions employed during the reaction but was frequently greater than 90%. Uracil was more readily removed than thymine or cytosine.

Verwoerd has investigated the reaction of hydroxylamine with the pyrimidine with the object of evaluating the potentialities of the reagent in the sequential analysis of nucleic acids. These studies have shown that the first step in the reaction is analgous to the reaction with hydrazine. (203)



This is followed by a replacement of the urea residue by hydroxylamine with formation of the oxime.

Reaction with cytosine or uracil leads to the same product. In each case reaction is carried out in aqueous solution under mild conditions which do not lead to severe degradation of the chain. The possibility of applying this method in the present work is limited by the necessity to remove the hydroxylamine from the oxime derivative. A possible approach to this is discussed later.

Experiments with chemically synthesised polyU.

In these experiments it was attempted to decide which of the methods described above was most effective in producing a ribose phosphate polymer from polyU. In addition attempts were made to determine, in each case, the optimum conditions for reaction if this was not already known and where possible modifications were introduced to minimise degradation of the product.

In each case the mixture after reaction was fractionated on SephadexG25 and the effluent fractions analysed for pentose (G.M.I) and reducing power (G.M.3)

It will be seen later that the method used to determine reducing power gives anomalous results with phosphoric acid esters of ribose and this has invalidated some of the results of these early experiments. They

are included here because they led to the investigation on uridine phosphates, described later, which produced some unexpected results.

The action of hydrazine.

Hydrazinolysis is normally carried out using the free base either in aqueous solution (199) or as the the anhydrous liquid (200) and at a temperature between 50°C and 90°C. In aqueous solution hydrazine is strongly alkaline and for this reason Takemura used the anhydrous liquid in order to avoid degradation of the RNA chain by alkaline hydrolysis. However, the use of anhydrous hydrazine at elevated temperatures would appear to favour the formation of hydrazones, a reaction which is undesirable for the present purpose.

In view of this it was felt justifiable to moderate the reaction conditions by employing aqueous solutions at a less destructive pH and a lower temperature. Therefore the reactions of hydrazine with polyU were performed at 37°C. The optimum pH for the reaction was determined in a manner resembling that used by Verwoerd et al. to determine the pH optimum of the reaction of hydroxylamine with UMP.

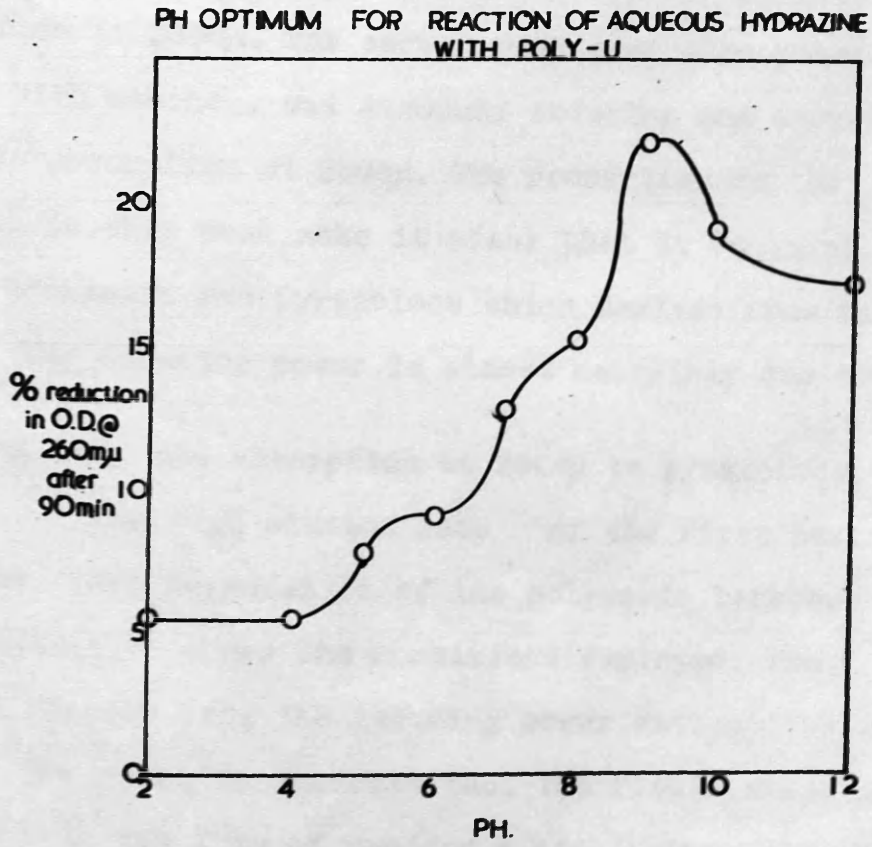
The method is described in detail in Expt. 41

Aqueous solutions of hydrazine(4.0M with pH varying from 2-12 were mixed with equal amounts of a standard solution of polyU. For each solution the optical density at 260mμ was determined immediately after mixing and then after 90min. Blank solutions were prepared in which the hydrazine (at the correct pH) was mixed with an equal volume of water.

The rate of removal of $D_{260}$ as a function of pH is shown in Fig.25

There is a clear peak in the region of pH9. In a larger scale experiment (Expt.42) polyU was dissolved in 10M hydrazine pH9 and incubated at 37°C. Measurement of the disappearance of optical density at 260mμ showed that reaction was complete after 2hrs. The solution was then applied directly to a column of SephadexG25 which was eluted with 0.2MNaCl [it is perhaps noteworthy that Takemura et al. (200) used ethanol precipitation to isolate the hydrazinolysis product of RNA. However, the product from polyU is not precipitated by ethanol]. The fractions collected from the Sephadex column were analysed for pentose (G.M.1) and reducing power (G.M.3). The optical

FIG. 25

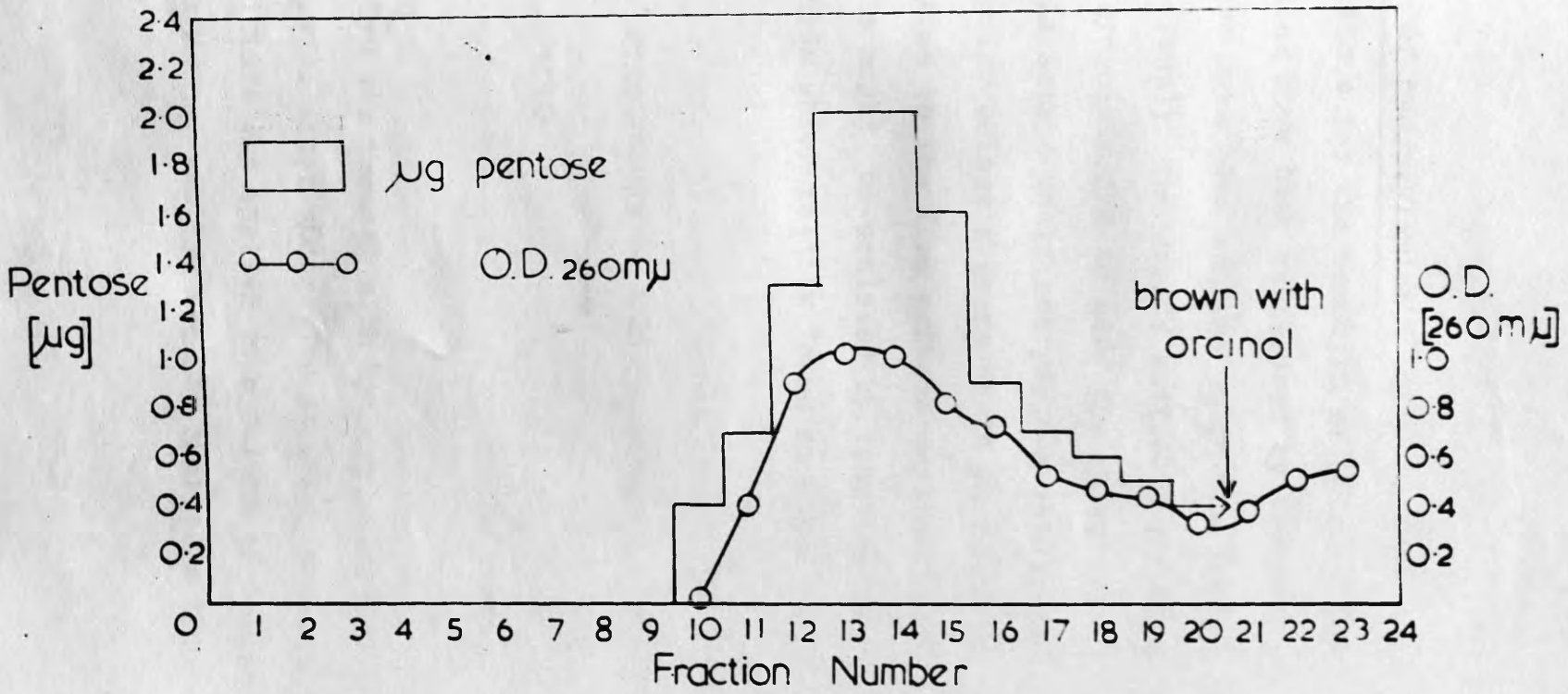

PH OPTIMUM FOR REACTION OF AQUEOUS HYDRAZINE WITH POLY-U
% reduction in O.D.@ 260mμ after 90min
0
5
10
15
20
2
4
6
8
10
12
PH.

density at 260mμ was also noted. These analysis (Fig.26) showed that material was eluted in two peaks. The higher molecular weight material which is eluted first, was orcinol positive (giving a green colour typical of pentose compounds), showed no reducing power and no specific absorption at 260mμ. The second peak gave a deep brown colour with orcinol, was strongly reducing and showed specific absorption at 240mμ. The properties of the material in this peak make it clear that it contains the excess hydrazine and pyrazolone which derives from the uracil. The reducing power is almost certainly due to hydrazine and the absorption at 240mμ to pyrazolone.

The high elution rate of the first peak indicates that degradation of the polymeric backbone is not extensive under the conditions employed. The negative results from the reducing power estimations were taken, at the time, to indicate that the ribose moieties were still in the form of ureides, a conclusion which was later shown to be justified although it is now known that the alkaline copper reagent gives abnormal results with compounds of this type.

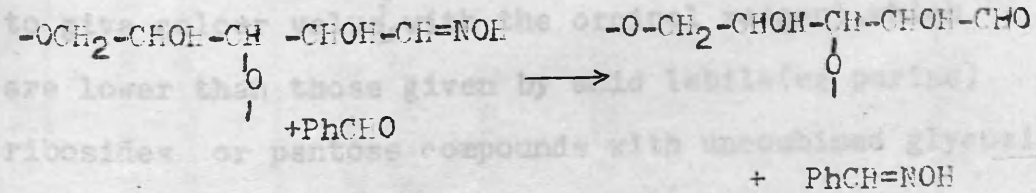
FIG. 26.

Hydrazine treated polyuridylic acid fractionated on Sephadex G25.



2) The action of hydroxylamine.

Optimal conditions for the reaction of hydroxylamine with uracil ribosides have been determined by Verwoerd et al. (203) and these have been adhered to in the present work. As mentioned previously the ureide initially produced reacts further with hydroxylamine to give the oxime and it was clear that this method would not yield a ribose phosphate polymer from polyU unless a means could be found to convert the oxime to the free pentose derivative. It was hoped that this might be achieved by treating the oxime with benzaldehyde which readily forms an ether soluble oxime:



PolyU was treated with hydroxylamine (IOM, pH 10) at 37°C (Expt. 43). After absorption at 260mμ was reduced to zero, the mixture was applied to a column of Sephadex G25 a and eluted in a manner similar to the hydrazine treated

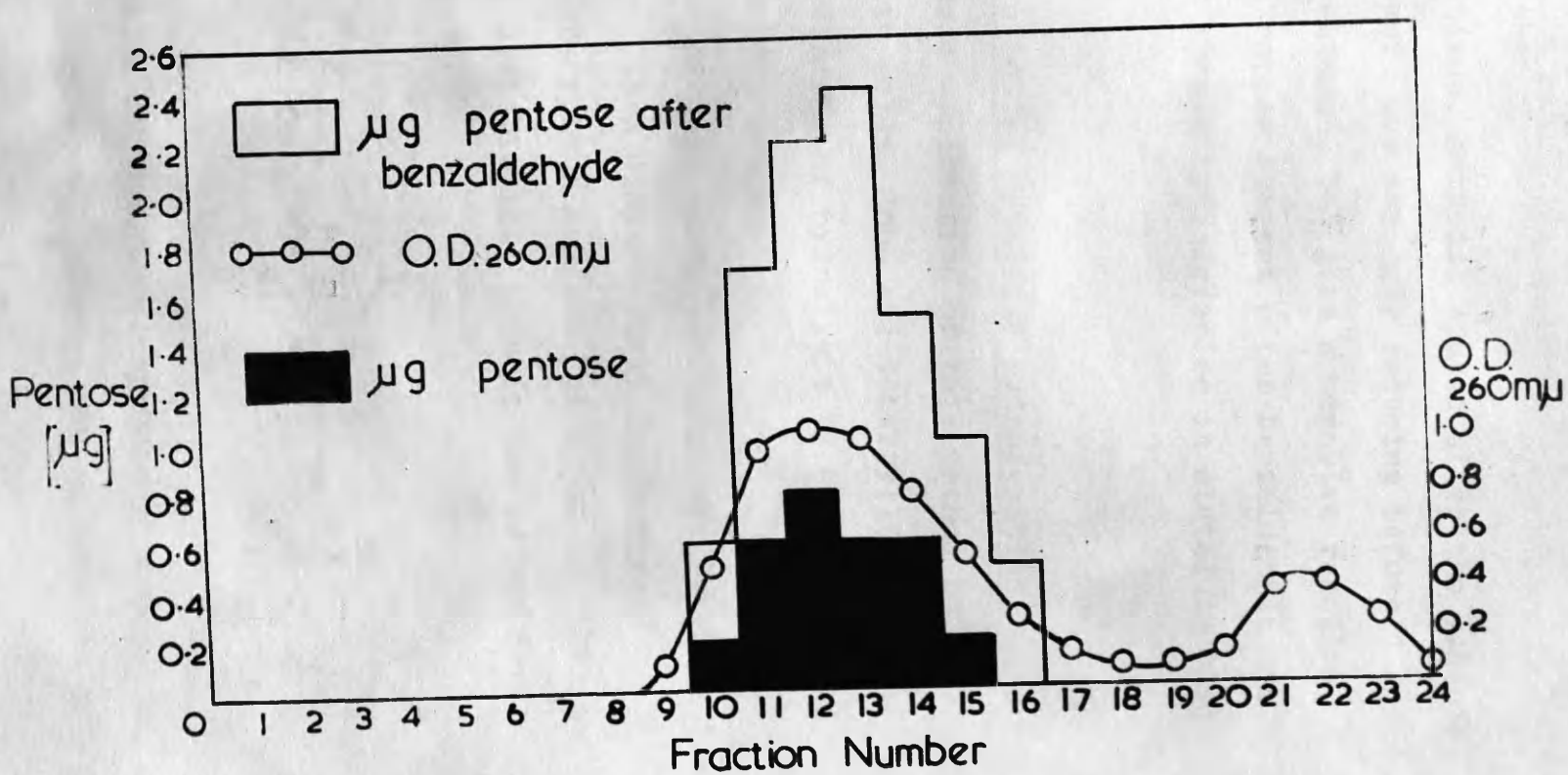
material. After analysis of the fractions (Fig27 ) each was treated with an excess of benzaldehyde in ether. This treatment was repeated and the solution freed of benzaldehyde and benzaldoxime by washing with ether. The fractions were then analysed (fig27).The outstanding feature of these analyses is the observation that after treatment with benzaldehyde the pentose content of ~~of~~ the peakl material,as measured by the orcinol method rises to a value more than double the original value.

This result would be expected if the material in peakl contained an acid stable riboside from which the aglycone was removed by benzaldehyde treatment. Stable ribosides such as pyrimidine ribosides are known(201) to give colour values, with the orcinol reagent,which are lower than those given by acid labile(eg purine) ribosides or pentose compounds with uncombined glycosidic ~~no~~ positions.

Negative results were again obtained on analysing the ~~peakl~~ material of peak 1 by the alkali copper method,both before and after benzaldehyde treatment.At the time this was taken to indicate that in neither case did the material contain ribose unsubstituted in the

FIG. 27.

Hydroxylamine treated polyuridylic acid before and after benzaldehyde treatment Fractionated on Sephadex G25.

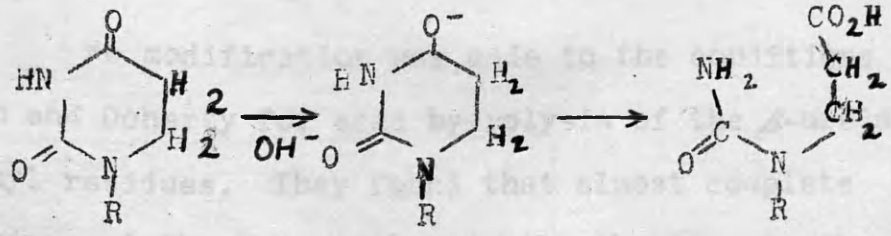


glycosidic positions. Material eluted after the pentose containing polymer was strongly reducing before benzaldehyde treatment, but gave a negative reaction with the alkali copper reagent after benzaldehyde treatment, indicating that excess hydroxylamine is eluted in this region.

3) Hydrolysis following catalytic hydrogenation.

In their application of this method to the isomeric uridylic acids, Cohn and Doherty(195) decided the optimal conditions of hydrolysis by elegant use of ion exchange chromatography.

In the present work, advantage was taken of the observation that dihydrouracil derivatives demonstrate an absorption maximum at 235mμ in alkaline solution. It is probable that this is due to the formation of an anion:-



Formation of the β-ureidopropionyl derivative by ring opening(see above) removes the chromophore and

this provides a convienent basis for measuring the extent of reaction. When alkaline cleavage is complete the optical density at 235mμ is zero.

It is fortunate that this means of following the reaction is available since prolonged treatment with alkali is likely to cause degradation of the phosphodiester linkage when applied to a polyribotide.

Cohn and Doherty (195 ) found that in 0.1N sodium hydroxide at room temperature dihydrouridylic acid (the 2'- or 3'- isomer) was converted to the corresponding β-ureido-propionyl ribotide in 1hr.

These conditions have been found, using the spectrophotometric method described above, to be optimal for alkaline cleavage of the dihydrouracil compounds derived from uridine-5'-phosphate and polyuridylic acid in the work described later.

No modification was made to the conditions used by Cohn and Doherty for acid hydrolysis of the β-ureidopropionyl residues. They found that almost complete conversion of the β-ureidopropionyl ribotides to the corresponding ribose phosphates is achieved by treatment with 0.1Nacid for 24hrs at room temperature.

Although the hydrolysis conditions necessary to remove dihydrouracil from its ribosides are mild, it might be expected that they would lead to serious degradation when applied to apolyribonucleotide. If this were so, the method would be precluded as a means of producing a ribose phosphate polymer.

Data on the rate of hydrolysis of RNA by acid and alkali are scarce (204), and to decide this issue it wasfelt necessary to apply the hydrolysis conditions in question to RNAand to see if the degradation was serious. Accordingly, low molecular weight RNA (commercial yeast RNA, L.Light and Co. Ltd.)was treated with 0.1N NaOH for 1 hr. and then with 0.1N HCl for 24 hr. Samples were withdrawn after each treatment and dialysed against 0.2MKCl. The optical density of the solution was measured before and after each treatment and these values show (Table 17) that only 4% of the RNA is rendered smallenough to pass through the dialysis membrane.

Table 17.
Treatment of RNAwith alkali and acid.

| Treatment | % RNA dialysable |
|---|---|
| None | 0.2 |
| 0.1N NaOH | 2.1 |
| 0.1N HCl | 4.0 |

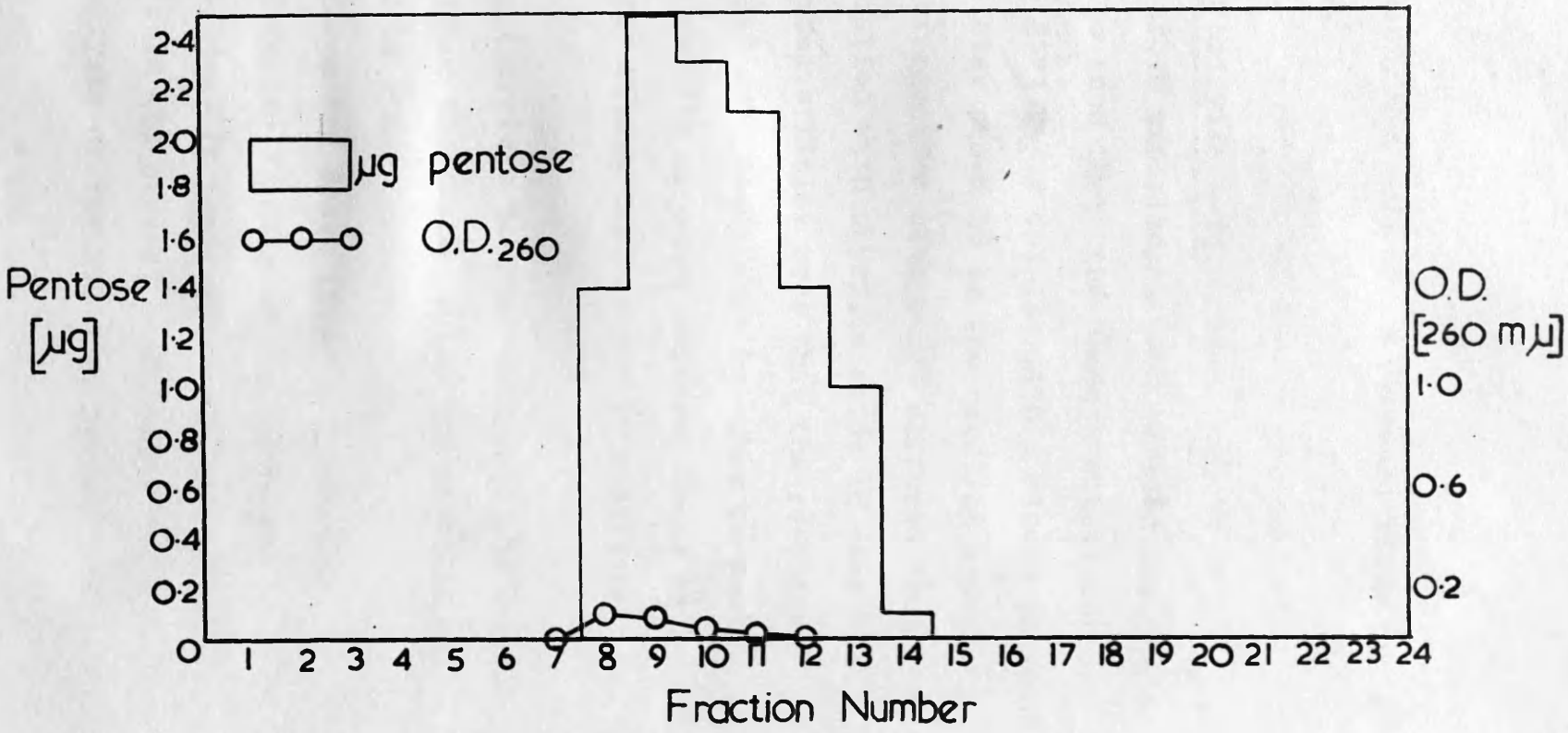
although this does not give a quantitative measure of the extent of degradation, it can be taken from the data of Michelson (189) that the 96% of the RNA which is retained after dialysis has an average chain length greater than 10-12. It seems unlikely, therefore, that application of the method of Cohn and Doherty to polyU will lead to objectionably extensive degradation of the chain.

In a small scale experiment (Expt.44) polyU (chem. synth. Sephadex fractionI) was hydrogenated over 5% rhodium on alumina until the optical density at 260mμ had fallen to zero. The reduced polymer was subjected to the appropriate treatment with alkaline and acid(Expt.44) and then fractionated on a column of SephadexG25 (G.M.18). Analysis of the effluent fractions (Fig28) showed a single peak, eluted early as polymeric material, which was orcinol positive,had negligible absorption at 260mμ and was negative to the alkali copper reagent.

From the results of the above series of experiments it was clear that all of the treatments applied to polyU gave rise to polymeric material which was free of uracil. However,the negative reaction of the product in each case, with the reagent for reducing sugars was taken to indicate that the glycosidic position

FIG. 28.

Polyuridylic acid treated by Cohn's method Fractionated on Sephadex G25.



of the ribose moiety was left in a combined state by by these methods.

Experiments with uridylic acid.

At the time the above experiments were carried out, there was no evidence to show that the Somogyi alkali-copper reagent does not give valid results with a ribose phosphate polymer, as was later shown to be the case. It appeared that the course of reaction with polyU differed from the course of reaction with urydilic acids in each case. The alternative possibilities were that the reaction conditions employed were unsuitable or that in fact, contrary to the results of other workers, these methods do not produce free ribose derivatives from uridine compounds.

It was therefore decided to repeat the experiments with a uridine phosphate using the reaction conditions reported by other workers.

Both Baron and Brown (199) and Cohn and Doherty (195) had relied largely on ion exchange chromatography to identify their products from uridylic acids 'a' and 'b'. In the present work, paper chromatography was chosen for analysis of the reaction products as it was

desirable to use an independent method. Uridine-5'-phosphate was chosen as test compound, because ribose-5'-phosphate was available for comparison. Interest was centred on two reactions, a) hydrazinolysis and b)hydrolysis following hydrogenation, since detailed reports were available of the production of ribose phosphates from uridylic acids using these methods.

1) The reaction of uridine-5'-phosphates with hydrazine.

In their experiments, Baron and Brown used hydrazine hydrate at 70°C and these conditions were adhered to in the present work. Isolation of the product for paper chromatography is facilitated by using salt free hydrazine which can be removed readily by a cation exchange resin.

If the hydrochloride were used ,as in the previous experiment with polyU, this would introduce a considerable amount of salt which interferes with the subsequent paper chromatography.

Uridine-5'-phosphate was dissolved in hydrazine hydrate and the solution was held at 70°C for ten minutes (Expt.44). After reaction, the solution was diluted and the hydrazine removed by treatment with an acidic cation exchange resin (G.M.22). The acidic product was neutralised

with lithium hydroxide, freeze-dried, purified as described in the experimental section and converted to the ammonium salt (G.M.22). Authentic R5P was applied to the chromatogram alongside the hydrazinolysis product. Applications of R5P and the hydrazinolysis product were also applied at one position on the chromatograms, which were developed in solvents 2 and 6.

It can be seen (Fig.29 ) that the main product of hydrazinolysis has a $R_f$ value corresponding to ribose-5'-phosphate in each solvent. Two other components are also revealed by the benzidine-TCA reagent.

To account for the formation of these additional products, two possibilities present themselves. First, they might arise from the alkaline degradation of R5P (see Appendix 3). Secondly they might represent the ureido derivative and the hydrazone of R5P. This pair of compounds could arise by a reaction similar to that demonstrated for the analogous reaction with hydroxylamine (203).

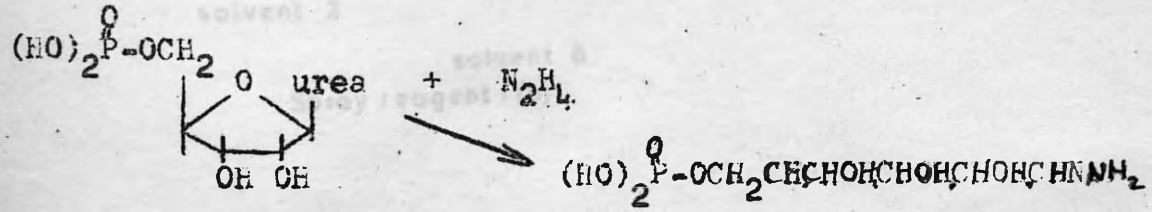
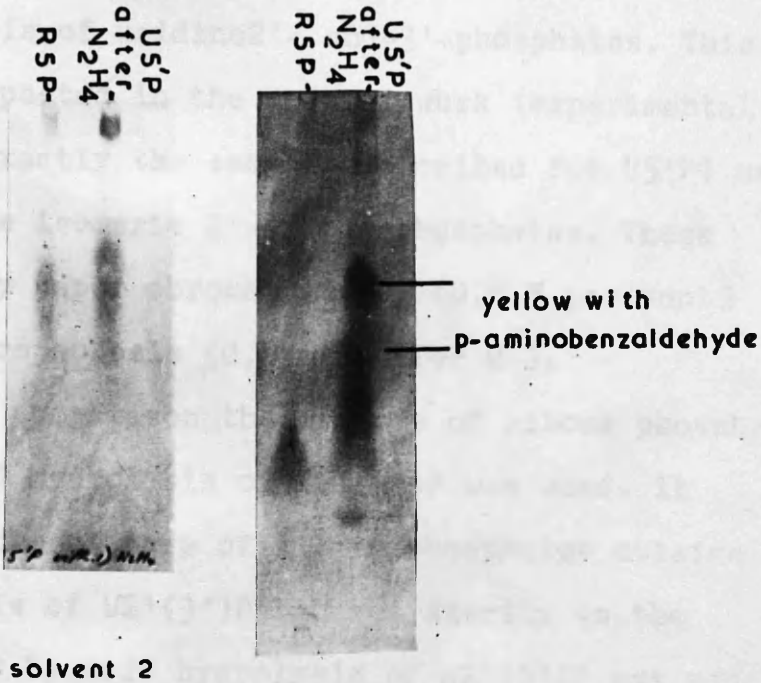


FIG. 29.

Paper chromatograms showing hydrazinolysis products of US'P




solvent 2

solvent 6

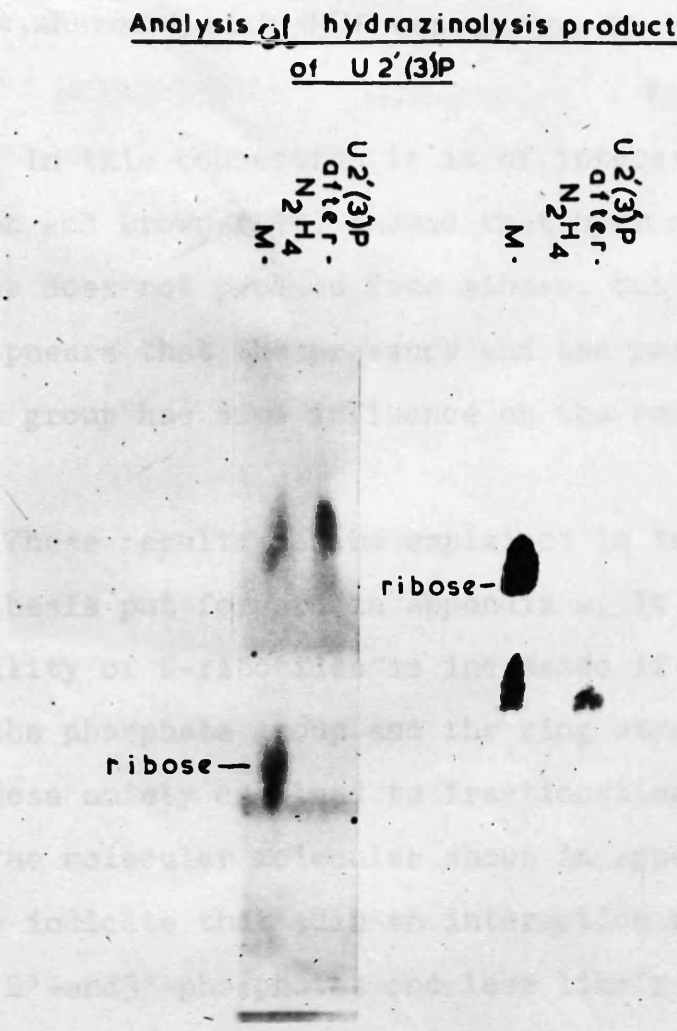
Spray reagent I (b)

To test the latter possibility, a similar set of chromatograms was treated with Ehblich's reagent(G.M.7) It was found that the two spots additional to R5P gave intense yellow colours, indicating the presence of-$NH_2$ groups. This observation was in accord with the proposal that these products correspond to the ureide and hydrazone of R5P.

It will be recalled that Baron and Brown (199) observed that only the ribose phosphates were produced on hydrazinolysis of uridine2'- and-3'-phosphates. This reaction was repeated in the present work (experimental procedure was exactly the same as described for U5'P) using a mixture of the isomeric 2'-and 3'-phosphates. These were analysed by paper chromatography (G.M.5 ;solvent 2 ) and paper electrophoresis (G.M.6 ;buffer 2 ).

For a comparison the mixture of ribose phosphate obtained by acid hydrolysis of A2'(3')P was used. It was found that the mixture of ribose phosphates obtained by hydrazinolysis of U2'(3')P behaved exactly as the mixture obtained by acid hydrolysis of A2'(3')P was made. spots in the latter mixture(marked M in Fig30) correspond to ribose and R4P produced by the acid treatment of A2'(3')P . Ribose is produced by further hydrolysis of the ribose phosphates and R4P by isomerisation ofR2P andR3P.

FIG. 30.




Paper - chromatography - electrophoresis
solvent 2 buffer 2

It seems then that the course of reaction of hydrazine with U5'P differs from that with U2'P and U3'P in that the aglycone is completely removed from the latter two compounds, whereas, with U5'P conversion to R5P is incomplete

In this connection it is of interest to note that Baron and Brown (199) showed that hydrazinolysis of uridine does not produce free ribose, but an N-riboside. Thus it appears that the presence and the position of the phosphate group has some influence on the course of the reaction.

These results can be explained in terms of the hypothesis put forward in Appendix A. It is proposed that lability of N-ribotides is increased if interaction between the phosphate group and the ring oxygen of the ribofuranose moiety can lead to fractionation of this oxygen. The molecular molecules shown in Appendix A (figs 35) indicate that such an interaction is possible with the 2'-and3'-phosphates and less likely with the 5'-phosphates. In the nucleoside, the phosphate group is absent. On this basis the expected order of stability of theN-riboside linkage is:nucleoside > 5'-ribotide $\gg$ 2'- and 3'- ribotide. This mechanism might also explain the results obtained by hydrazinolysis of polyU(later section)

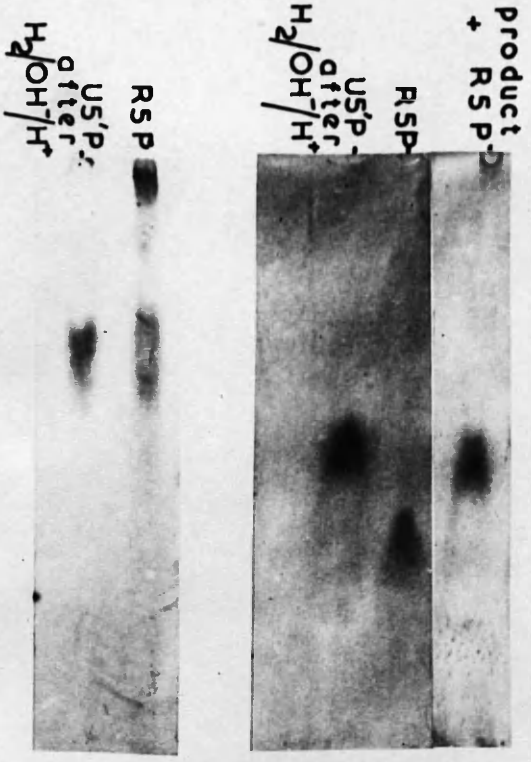
2) Hydrolysis following catalytic hydrogenation of uridine-5'-phosphate.

The conditions used in this experiment (Expt47) were essentially those described by Cohn and Doherty for the treatment of uridine-2'-and3'-phosphates. A slight modification was introduced in the working up procedure in order to avoid excessive introduction of salts which would interfere with paper chromatography. After alkaline hydrolysis of the dihydrouridine-5'-phosphate, the solution was neutralised by the addition of HCl. Instead of adding more HCl to bring the solution to0.1N with respect to hydrogen ions, the same result was achieved by adding excess of a cation exchanger in the hydrogen form, to replace the sodium ions.

After acid hydrolysis the lithium salt was prepared as described in the experimental section, and converted to the ammonium salt for chromatography. Paper chromatography of the product (G.M.5,solvents2and 6 Fig31 ) showed a single reducing spot. In solvent 2 the $R_f$ value of the product is identical to that ofR5P. In solvent 6 the product is apparently slower than R5P, but when the two are co-chromatographed a single spot is obtained which indicates that the slower $R_f$ is due

FIG. 31.

**Paper chromatograms showing product from U5'P treated by Cohns method**




solvent 2 solvent 6

Spray reagent I(b)

to the presence of salt.

From the spectrographic data it is clear that catalytic hydrogenation and alkaline hydrolysis go to completion. The paper chromatograms show that R5P is the sole ribose containing product after acid hydrolysis, in agreement with the results of Doherty and Cohn who studied these reactions on the 2' and 3' isomers.

Reappraisal of the results with polyU in the light of results with uridine phosphates.

At this stage in the work the author was faced with an apparent anomaly. Early experiments with polyU, it will be recalled, provided a material which was non-reducing, according to the alkali-copper reagent, with every base-removal method tested. Yet with the uridylic acids one of these methods viz. that of Doherty and Cohn, produced the corresponding ribose phosphate quantitatively, while another, reaction with hydrazine produced only ribose-5-phosphate, to a considerable extent.

Two alternative explanations might account for these observations. 1)the glycosidic linkage in the case of

the monoribotides might be less stable than the corresponding linkage in the polyribotides. Such a difference can be accounted for in terms of the mechanism proposed in Appendix A by assuming that, in the polyribotides, interaction of the phosphodiester with the ring oxygen is limited by steric hindrance.

2)The methods might be succesful in removing the bases from polyU, and if this is so it is necessary to propose that the products, though possessing free glycosyl ribose moieties do not reduce the alkaline copper reagent.

Observations on the Alkaline copper method as a means of determining the reducing power of pentose phosphates.

Since results obtained using the alkaline copper reagent on the polymeric phosphate derivatives of ribose were open to question, it was decided to test the method on a pure substance (ribose 5-phosphate) which was more closely related to these compounds than ribose, which was normally used in standardising the procedure (G.M.3).

Solutions of ribose and ribose-5-phosphate were prepared and aliquots were analysed for pentose content

using orcinol reagent (G.M.1). From these results the volumes of the solutions were adjusted so that the concentration of pentose in each was the same. From each solution, increasing volumes were withdrawn and tested for reducing power as described in G.M.3). When the colour values obtained were plotted against the volume of solution taken, it was found that a) the ribose gave a linear relationship b) the ribose-5-phosphate gave a series of points through which it was difficult to draw a straight line, or a simple curve. Furthermore, the colour values produced by ribose-5-phosphate were much lower (approx 0.5x) the values obtained from ribose.

It seemed likely that these anomolous results might be caused by the degradation of theR5P under the alkaline conditions used in the Somogyi-Nelson method. The instability to alkali of R5P, first recognized by Khym et al. (202) is discussed in AppendixB.

Under conditions intended to simulate those employed in the alkaline-copper method (0.1NNaOH,100°C) it was found (Expt.48) that the pentose in ribose-5-phosphate was almost completely destroyed, as judged by the orcinol reaction, after 15min. Destruction of pentose was accompanied by a somewhat slower release of

inorganic phosphate. Ribose is not so unstable to alkali (Appendix B) and it appears probable, therefore, that the observed differences between the behaviour of ribose and R5P to the alkaline copper reagent can be explained by the extreme sensitivity to alkali of R5P.

This result cast further doubt on the validity of the earlier observations on the products from polyU, in which free glycosidic positions were tested for by the alkaline copper method.

It was also clear that any method used to determine the reducing power of these polymers should not involve the use of alkali. In addition, the use of acidic reagent must be avoided, because treatment with acid might produce reducing compounds from polyribotides in which the glycosidic position is substituted ba an acid labile group ( for instance the $\beta$-ureidopropionyl group).

A new method for the estimation of pentose compounds unsubstituted in the glycosidic position.

Since sodium borohydride reacts specifically with free aldehydic groups, including the glycosidic positions of sugars , it was decided to investigate the possibility of using this reagent in the present problem.

Ribose derivatives with free glycosidic positions are reduced quantitatively to the corresponding ribitol

derivatives by sodium borohydride, under mild conditions. It was expected that the ribitol compounds formed would not give the green colour characteristic of pentose, with the ferric orcinol reagent. This expectation was borne out by a preliminary qualitative experiment, which was followed by thorough quantitative investigation (See G.M.23 ).

Two series of solutions were prepared in Tris buffer each series containing increasing quantities of R5P. The solutions in one series were treated with similar quantities of sodium borohydride, added to each tube as the solid, and after remaining overnight at room temperature, the two sets were treated with the ferric-orcinol reagent (G.M.1). In Fig.33 are shown the two curves (AandB) corresponding to the colours obtained before and after borohydride treatment, as a fraction of the amount of ribose-5-phosphate (expressed as volume of standard R5P solution). Curve3 shows, that after borohydride treatment, ribose-5-phosphate still produces a colour with the orcinol reagent.

It is apparent that this colour (henceforward referred to as"residual colour") is not due to pentose, for it is grey. Pentose compounds give a characteristic green or blue colour with the orcinol reagent. Furthermore, the

absorption at 660mμ of the "residual colour" bears a linear relationship to the amount of ribose-5-phosphate taken and it seems likely that it arises from theproduct of reduction. The linear relationship makes it easy to take the "residual colour" into account in calculating the unsubstituted pentose from the orcinol colours.

The method is described in detail in G.M.23 and its application to polyribotides obtained from polyU by removing the bases is discussed in the next sub-section.

## A reinvestigation of the removal of uracil from polyuridylic acid by a)hydrazinolysis andb)Cohn's method

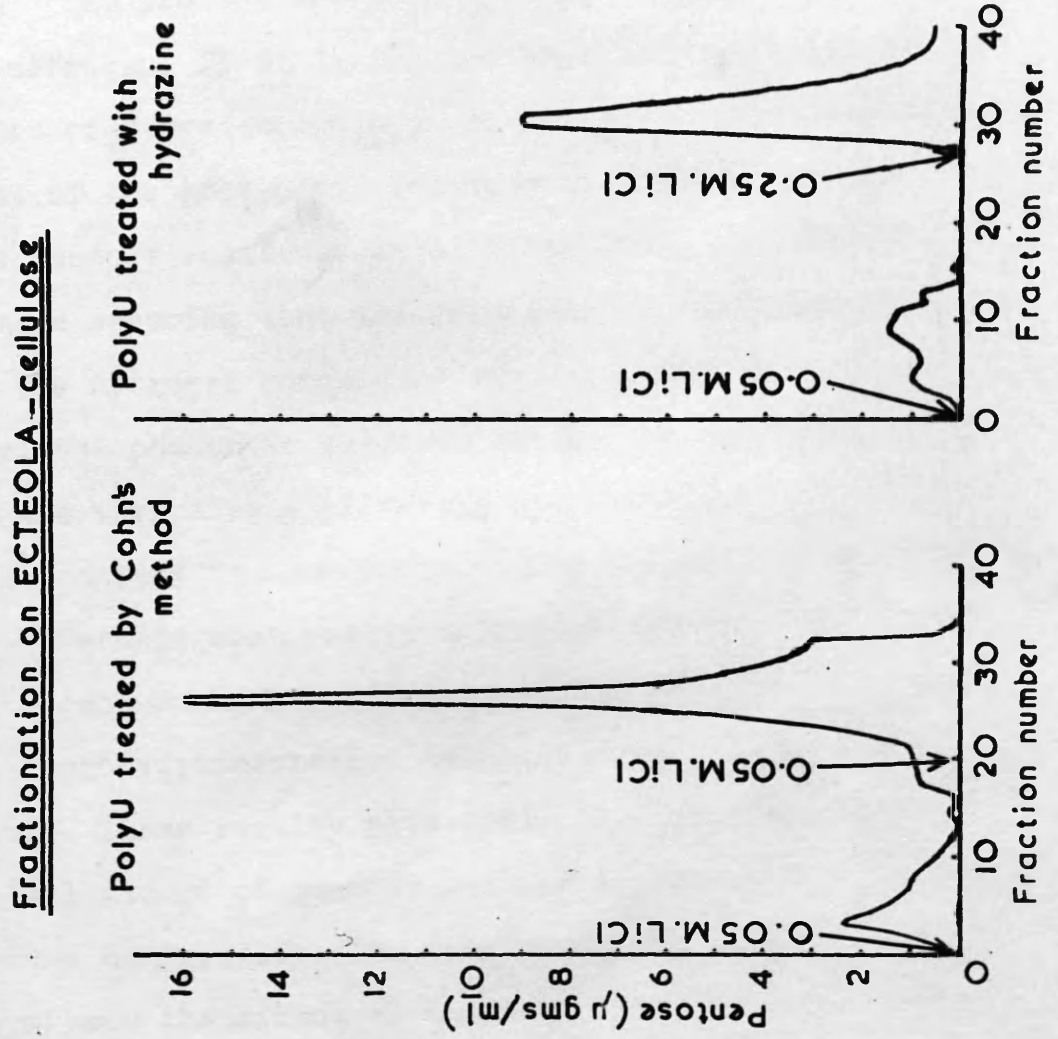
The two reactions which were to be reinvestigated were carried out as described previously (Expts. 42 and 44) on a larger (one hundredfold) scale and are described in Expts. 49,50 and 51. The isolation procedure, however, was modified. Previously the products were isolated by gel filtration on SephadexG25. In these experiments (Expts. 49 and 51) the products were isolated by ion exchange chromatography on ECTEOLA-cellulose (G.M.19). This method was chosen

because it gave a better separation of the polymers from low molecular weight compounds than gel-filtration and was more likely to remove hydrazine, which might possibly form salt linkages with the polymer. After application of the products to ion exchange cellulose, and a preliminary wash with water, elution was continued with solutions of lithium chloride, whose concentrations were chosen so that the first solution would remove all material of low molecular weight (including degraded polymer smaller than tetramer in addition to hydrazine and β-ureidopropionate). The second solution was of high concentration (0.5M) and eluted the polymers entirely in a few fractions. In each case the elution pattern (Fig 32a. and b) show that degradation is low, though it is clearly greater by the Cohn's method than by hydrazinolysis.

The lithium salts of the polymers were isolated from the excess of lithium chloride by leaching the freeze-dried elutes with organic solvents and samples were submitted for estimation of nitrogen by a micro-combustion method (the author is grateful to Mr J.M.L. Cameron for performing these analyses).

The product of hydrazinolysis contained 6.43%

FIG. 32.a and b.

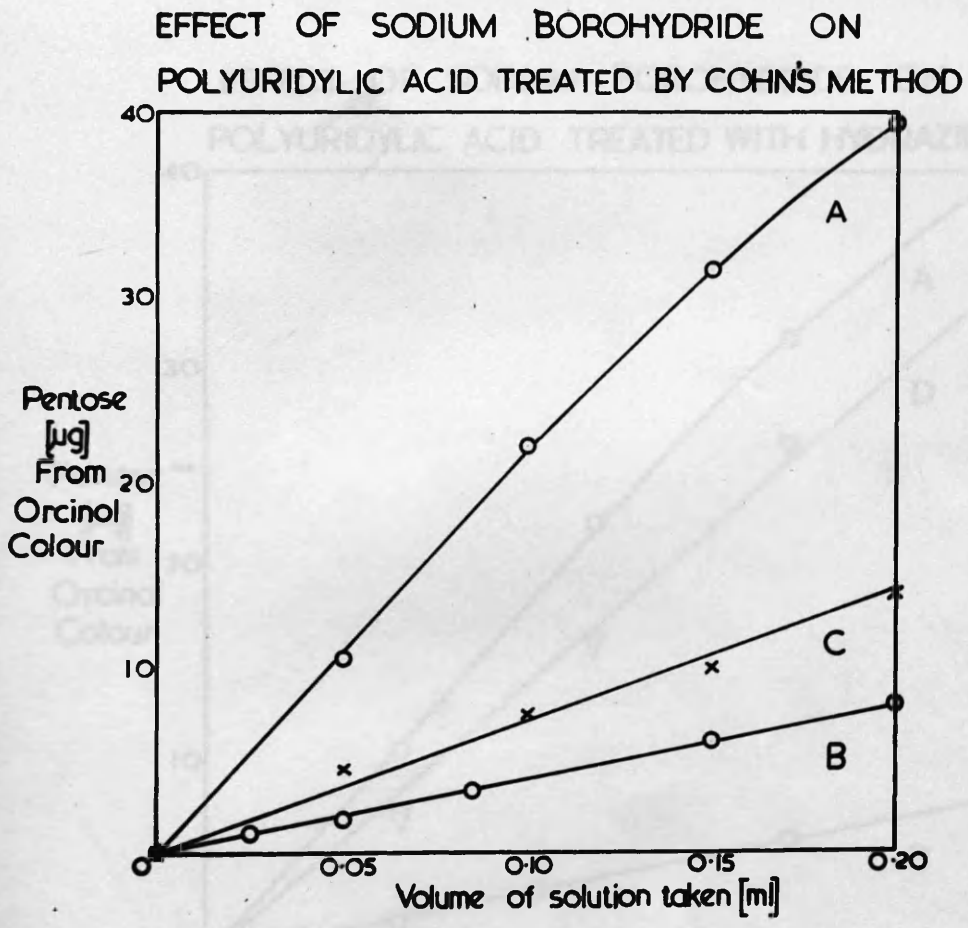

Fractionation on ECTEOLA-cellulose
PolyU treated by Cohn's method
PolyU treated with hydrazine
Pentose (μ gms/ml)
Fraction number
0.05M.LiCl
0.25M.LiCl

nitrogen. If it is assumed that this nitrogen all arises from urea, the urea comtent of the product corresponds to 50.6 moles per cent of the pentose residues.

The product obtained by Cohn's method contained 1.94% nitrogen. If it is assumed that this nitrogen is in the form of β-ureidopropionyl residues, the ureidopropionyl content of the product corresponds to 14.6 moles per cent of the pentose residues. Both of the above calculations were made assuming that the remainder of the compound (other than the nitrogen containing residues) was the lithium salt of a ribose phosphate polymer. Values for %nitrogen residues which are very little different are obtained if the total pentose content is estimated using the orcinol method(G.M.1)

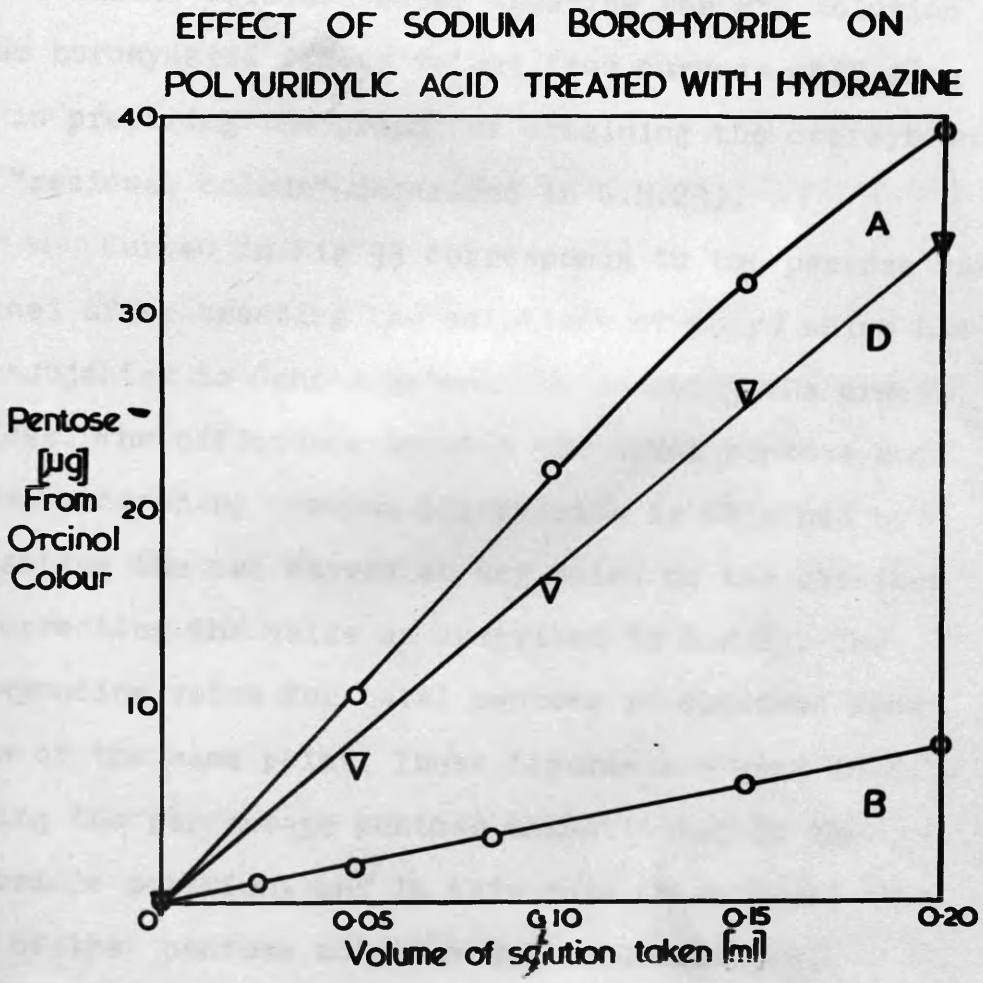
Perhaps more easily interpretated than the results of nitrogen estimation are the results obtained when the sodium borohydride/orcinol method was applied to these polymers. These results give a direct comparison between the total amount of pentose and the pentose which is reduced by sodium borohydride. The results illustrated in Figs 33 and34 show the effect of treating solutions containing the polymers with sodium borohydride. Solutions of the two polymers were prepared so that the pentose concentration in each case was similar to the R5P solution used in preparing the standard graph for correcting for "residual

FIG. 33.




A - before $NaBH_4$ treatment
C - after " "

FIG. 34.




A - before $NaBH_4$ treatment
D - after " "

colour" (SeeG.M. 23) The curve A in both Figs33 and 34 corresponds to the pentose concentration obtained from the orcinol analysis of the solutions of R5P, and polyuridylic acid which had been treated with hydrazine (Fig 34 ), or Cohns method (Fig. 33) , before borohydride treatment.

CurveB in Figs 33 and34 corresponds to the pentose values obtained after treating the R5P solution with sodium borohydride (These values,from curvesA andB were used in preparing the graph for obtaining the correction for "residual colour" described in G.M.23).

CurveC in Fig 33 corresponds to the pentose values obtained after treating the solutions of polyU which had been subjected to Cohn's method for removing the uracil residues. The difference berween the total pentose and pentose reduced by sodium borohydride is obtained by subtracting the two curves at any point on the abscissa and correcting the value as described in G.M.23. The corresponding value for total pentose is obtained from curveA at the same point. These figures are used in calculating the percentage pentose unsubtituted in the glycosidic position, and in this case it is found that 83.7% of the pentose moieties are unsubstituted.

Similarly curveD in Fig34 corresponds to pentose not reduced by borohydride in polyU which has

been subjected to hydrazinolysis. Calculated as described above the pentose unsubstituted in the glycosidic position in this case is 18.6% of the total. The linearity of the curves obtained with hydrazine treated polyU and polyU treated by Cohn's method shows that the orcinol colour obtained after borohydride treatment is independent of the initial concentration of the test compound within the practical limits of the orcinol test. Thus, the method described in G.M.23, using only one concentraton of the test compound, can be considered reliable.

The analysis of the product from polyU by treatment with Cohn's method are in good agreement. From the nitrogen estimation the apparent proportion of β-ureidopropionyl residues remaining is 14.6%. From the sodium borohydride method, the proportion of ribose moieties which are unsubstituted in the glycosidic position is 83.7% (i.e. 16.3% substituted). Agreement between the analyses is not so good for the product of hydrazinolysis. Calculated from the nitrogen content, the proportion of aglycone remaining (assuming it is urea) is 50.6%. The proportion of pentose unsubstituted in the glycosidic position is 18.6% of the total, according to the sodium borohydride analysis (i.e. 81.4% substituted). Because

calculation of the results from the nitrogen analysis involves assumptions regarding the nature of the aglycone and the purity of the product, the results obtained by the application of the sodium borohydride method are considered more reliable.

Application of Cohn's base re-moval method to polyU synthesised enzymically.

The results obtained with chemically synthesised polyU indicate that for the present purpose, the hydrogenation procedure produces a ribose phosphate polymer which is much more satisfactory than the hydrazinolysis product of polyU. Although hydrazinolysis is much simpler and causes less degradation of the chain, it leaves approximately four times as many glcosyl residues on the ribose moieties as the hydrogenation process.

It was now intended to submit the enzymically synthesised polyU to hydrogenation followed by hydrolysis to obtain a ribose phosphate polymer which was linked

through 3'-5'phosphodiesters only and which was of higher molecular weight than that obtained from polyU prepared by chemical synthesis.

In a small scale experiment (Expt.52) polyU prepared in Experiment 38 was reduced smoothly by hydrogenation over 5% rhodium on alumina at pH3.0. Subsequent treatment with alkali reduced the optical density at 235mμ to zero in 20mins. at room temperature.

An attempt to hydrogenate the polyU from the large scale preparation (Expt.39) produced no significant reduction as judged by removal of optical density at 260mμ, even after several days. Further additions of catalyst were made in an attempt to overcome possible catalyst poisoning and in fact this did cause a reduction in the optical density at 260mμ. It was shown by total phosphate determinations that the effect in this large scale experiment was due to the removal of the polymer either by absorption or by precipitation with the catalyst and not due to reduction of the bases.

It seems likely that failure to reduce this sample of polyU is caused by a contaminant which inhibits the catalyst. The alternative possibility, that the chain length of the polyU is too high to permit interaction

with the catalyst surface is improbable because the conditions used in the hydrogenation ( several days at pH 3) would almost certainly lead to extensive degradation of the chain and hence, after a prolonged period, the reduction should eventually have proceeded, albeit on degraded material.

Cohn and Doherty noted (195) that catalytic hydrogenation of pyrimidine derivatives is inhibited by the presence of small amounts of barium or adenine and the present writer has found that lithium ion totally inhibits reduction. It is well known that many catalysts are subject to poisoning by sulphur compounds. Attempts were now made, based on these observations, to remove possible inhibitors from the polyU preparation (Expt. 53).

In order to remove traces of protein, a possible source of sulphur compounds, the bentonite treatment used in the original preparation of the polyU was repeated and this was followed by a treatment with chloroform. To ensure the preparation was free of barium, lithium and free purines, the solution was passed through a column of cation exchange resin in the hydrogen form (G.M.22). Lest other low molecular weight inhibitors, which were not removed by the above tratment, might be present, the material was again fractionated on SephadexG25. (G.M.18).

After each treatment the solution was adjusted to pH3.0, fresh catalyst was added and the mixture shaken with hydrogen. In no case was the optical density at 260mμ reduced. An attempt to reduce the polymer under 5atmospheres of hydrogen was also fruitless.From this it was concluded that the most likely cause of inhibition is the presence in the polyU of purines which have been incorporated into the polynucleotide during the polymerisation of UDP, from RNA present in the enzyme preparation.

The nature of the new polymers.

Application of Cohn's method, for the removal of uracil residues, to polyuridylic acid, has produced three new polyribotides related to RNA.

Polydihydrouridylic acid was obtained by catalytic hydrogenation of polyuridylic acid over rhodium on alumina. This product showed the characteristic absorption of dihydrouracil derivatives at 235mμ, in alkali, which is quickly destroyed by alkaline cleavage of the dihydropyrimidine ring.

Poly β-ureidopropionylribotide was produced by alkaline cleavage of the dihydrouracil residues on polydihydrouridylic acid.

A ribose phosphate polymer corresponding to the "back-bone" structures of RNA was produced by mild acid treatment of poly β-ureidopropionylribotide. In this product 80 to 85% of the ribose moieties are unsubstituted in the glycosidic position. The ribose phosphate polymer derived from enzymically synthesised polyU (in which the phosphodiester linkages are exclusively 3'-5') has not been fully characterised because insufficient material was available. However, the first two stages of the preparation, catalytic hydrogenation and alkaline hydrolysis, when applied to a small sample of enzymically synthesised polyU showed results similar to those

obtained with polyU synthesised chemically (in which the phosphodiester linkages are both 2'-5 and 3'-5'). It appears likely that the last stage in the preparation of the ribose phosphate polymer, viz., acid hydrolysis of β-ureidopropionyl residues, will be similar with materials derived from both types of polyU.

The nature of the phosphate diester linkages in the new polymers is no doubt similar to that in the parent polyU. Thus, in the polymers derived from chem. synth. polyU, a mixture of 2'-5' and 3'-5' linkages is expected. In polymers derived from enzymically synthesised polyU, exclusively 3'-5' phosphodiester linkages are expected.

Accurate measurements of the molecular weights of the various products have not been attempted. However, the elution behaviour of the ribose phosphate polymer derived from chem. synth. polyU on gel filtration and on ion exchange chromatography on ECTEOLA cellulose show that degradation caused by the three processes leading to its formation from polyU, is not extensive. It is to be expected that products of correspondingly higher molecular weight arise by treatment of enzymically synthesised polyU, in which the degree of polymerisation is far greater than in polyU synthesised chemically.

The products obtained when polyU was treated with hydrazine or hydroxylamine to remove the bases, are not so well characterised as those obtained by application of Cohn's method.

Hydrazine treatment gave rise to a polyribotide in which approximately 80% of the ribose moieties remain unsubstituted by an unidentified aglycone. Degradation of the phosphodiester linkage was slight under the conditions employed.

The action of hydrazine and hydroxylamine on RNA have been studied by other workers, with a view to applying these reactions in structural studies of RNA (200, 203). The products from RNA have not been well characterised in either case and it is assumed by these workers that the action of the reagents on the bases of RNA is analogous to that observed in the mononucleotides. The application of hydrazinolysis has been shown in the present work, to lead to different results with mononucleotides and polynucleotides. It seems that studies of the actions of these reagents, on polynucleotides containing a single pyrimidine base would provide a more reliable basis for comparison of their action on RNA.

## The action of ribonuclease on polydihydrouridylic acid.

The products obtained from polyuridylic acid by the application of Cohn's method are now sufficiently well characterised to permit their use as model compounds in studies relating to RNA. A study of the action of pancreatic ribonuclease on polydihydrouridylic acid, described below, illustrates one a application for polymers of this type, in the study of mechanisms of enzyme action.

The rate of hydrolysis of chem. synth. polyU by pancreatic ribonuclease was compared with the rate of hydrolysis of the corresponding dihydrouracil derivative. Digests of the two polymers were set up (Expt.54). The extent of degradation was estimated by withdrawing aliquots at appropriate digest ion time, applying them to ECTEOLA-cellulose (G.M.19) and eluting the degraded material with 0.05M lithium chloride solution. Degradation products from polyU were estimated by measuring the optical density at 260mμ of the eluates, and products from polydihydrouradylic acid were measured by the orcinol method(G.M.1).

The results (table 18) show that both polymers are degraded by the enzyme. This observation is in agreement with the result of Witzel who found(204) that dinucleotides

containing dihydrouracil were hydrolised by ribonuclease. However, it was found in the experiment described here, that reaction with polyU is much more rapid (approximately ten fold) than with polydihydrouradylic acid. The relevance of this observation to the mechanism of ribonuclease action proposed by Witzel (204) is discussed in AppendixC.

It will be noted that degradation of the polymers is incomplete even after prolonged digestion with ribonuclease. This is in accord with the known specificity of ribonuclease for 3'-5' linkages. The polymers used contained both 2'-5' and 3'-5' internucleotidic linkages.

Table 18

Comparison of the rates of hydrolysis of polyU and polydihydrouridylic acid by pancreatic ribonuclease.

| Digestion time (hours) | % reaction | |
|---|---|---|
| | polyU | polydihydroU |
| 0.0 | 4.9 | 8.0 |
| 0.25 | 9.2 | 9.2 |
| 0.5 | 17.4 | 10.3 |
| 0.75 | 20.8 | 10.2 |
| 1.25 | 37.6 | 11.6 |
| 1.75 | 34.7 | 11.8 |
| 2.5 | 40.8 | 12.8 |
| 48 | 45.0 | 33.2 |

## APPENDIX

## Mechanism involved in the hydrolysis of nucleotides and polynucleotides.

### A The acid hydrolysis of N-glycosides.

Original proposals for the mechanism of acid hydrolysis of N-glycosides were put forward by Kenner (206). He suggested that for hydrolysis of the N-glycosidic linkage to take place at an appreciable rate a proton must become attached to the ring oxygen of the sugar.

From this he makes the following deductions:

a) if the proton adds to the aglycone (e.g. purine or pyrimidine), hydrolysis is facilitated if transfer of the proton from the aglycone to the ring oxygen is possible.

b) if the proton adds to the aglycone, and transfer to the ring oxygen is not possible, there is still a possibility of adding a second proton to the ring oxygen, if the positive charge on the glycone is distributed among other nitrogens in the aglycone.

In support of these proposals he draws comparisons between the stabilities to acid of glycosides of many types including those derived from purines, pyrimidines, imidazoles,

and various amines. For instance, the much higher lability of purine ribosides to acid hydrolysis than the corresponding pyrimidine ribosides can be explained in terms of point(b). Thus, the positive charge arising from protonation of N-9 of the purine can be expected to be more widely distributed over the other nitrogens of the base than a positive charge on a pyrimidine arising from protonation of N-3.

Dekker (208) has emphasised the importance of the first proposal and pointed out that amino substituents in C2 of a pyrimidine and C5 of an imidazole ring lie in close proximity to the ring oxygen of the sugar, in ribosides of these compounds. Similarly, N3 of a purine lies close to the ring oxygen. He proposes that in these cases transfer of a proton from the nitrogen atom to the ring oxygen is possible. In support of this he cites the observed higher lability to acid of the ribosides of imidazoles which carry 5-amino substituents over those possessing no substituents.

The apparent effect on the stability of ribose, of the presence, position and type of a phosphate ester group has been discussed in section III. These differences can be explained on the basis of Kenner's mechanism if it is possible for the phosphate group to influence the protonation

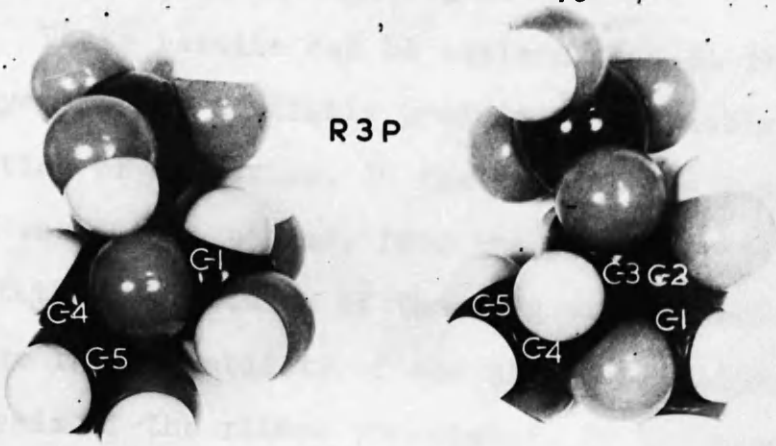
of the ring oxygen. Molecular models of R5P and R3P(Figs 35 a and b) show that for R5P and R3P the proton on the phosphate group can readily approach the ring oxygen by formation of a seven-membered ring.

For R3P however, three of the atoms involved in formation of the seven-membered ring are already fixed relative to each other in the ribofuranose ring. This may serve to increase the possibility of proton transfer to the ring oxygen from a phosphate group in the 3-position. For R5P only two of the seven atoms in question are members of the ribofuranose ring and the phosphate group is more free to attain positions at a distance from the ring oxygen. Because of this transfer of a proton from the 5-phosphate group to the ring oxygen might be less likely than transfer from a 3-phosphate group. A phosphate group in the 2-position bears a similar relationship to the ring oxygen as a phosphate group in the three position.

Baron and Brown (199) observed that the reaction of hydrazine on uridine gave rise to a product containing nitrogen, no ribose was formed. however the reaction withU 2P or U3P gave rise to the corresponding ribose phosphate. In sectionIII of the present work, evidence is presented which

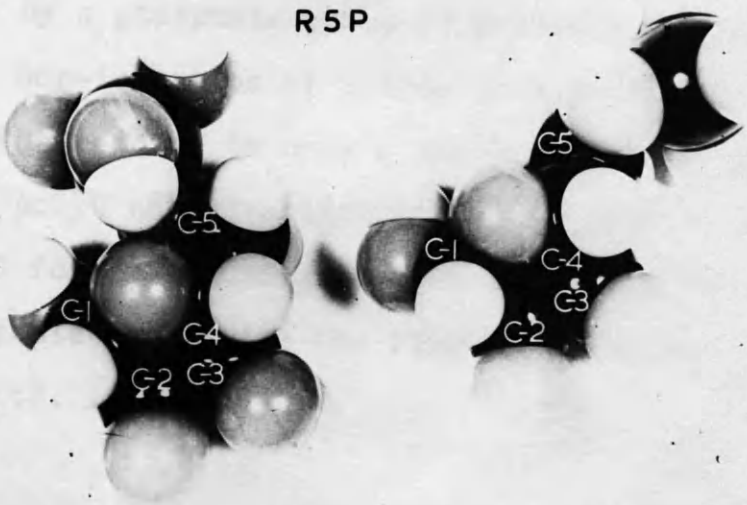
FIG. 35,a andb.

Molecular models showing interaction of phosphate with ring oxygen




Closest approach of phosphate to ring O

Phosphate position furthest from ring O



supports the results of Baron and Brown in that the product of hydrazinolysis of a mixture of U2P and U3P was apparently a mixture of ribose phosphates. Hydrazinolysis of U5'P however, gave a mixture of ribose-5-phosphate with compounds which apparently contained nitrogen.

These results can be explained if it is assumed that a glycoside is initially produced (presumably ureide) by the action of hydrazine. In the case of the 2-and 3-phosphates it would be expected, from the proposals discussed above that the protonation of the ring oxygen would cause an increase in the lability of the glycosidic linkage leading to hydrolysis of the ribose phosphates. In the case of the 5-phosphate a weaker interaction with the ring oxygen is expected and this might explain the incomplete conversion to R5P of this compound. In uridine no protonation of the ring oxygen by a phosphate group is possible and this might explain hhe non-formation of ribose on hydrazinolysis of uridine

The removal to only a small extent of glycosidic groups from polyU on hydrazinolysis, described in SectionIII is accounted for by assuming that in this case the interaction of the phosphate group with the ring oxygen is hindered by steric effects.

B The stability to alkali of the ribose phosphates.

Detailed observations on the effects of alkali on the isomeric phosphate esters of ribose were reported by Khym et al. (202) who found that the rate of phosphate release by 0.01N sodium hydroxide at room temperature followed the order R5P>R3P>R2P. These differences cannot be explained by simple hydrolytic mechanisms, since phosphate release was accompanied by destruction of the pentose moiety. Ribose occupies a position between R3P and R2P with respect to its rate of destruction by alkali.

The above authors proposed that rapid destruction of R5P, R3P and ribose proceeds via oxidation of ene-diols, which are formed by transfer of a proton from a carbon atom to the aldehydic oxygen. In R5P, and ribose formation of both 2,3-and3,4,-ene-diols is possible. In R3P, formation of the 2,3-ene-diol is possible but formation of the 3,4-ene-diol is prevented by the presence of the phosphate group. In R2P the formation of both 2,3- and 3,4-ene-diols is prevented by the phosphate group. Thus the difference in stability to alkali of the ribose phosphates are readily explained by this mechanism. However, it is difficult to account for the

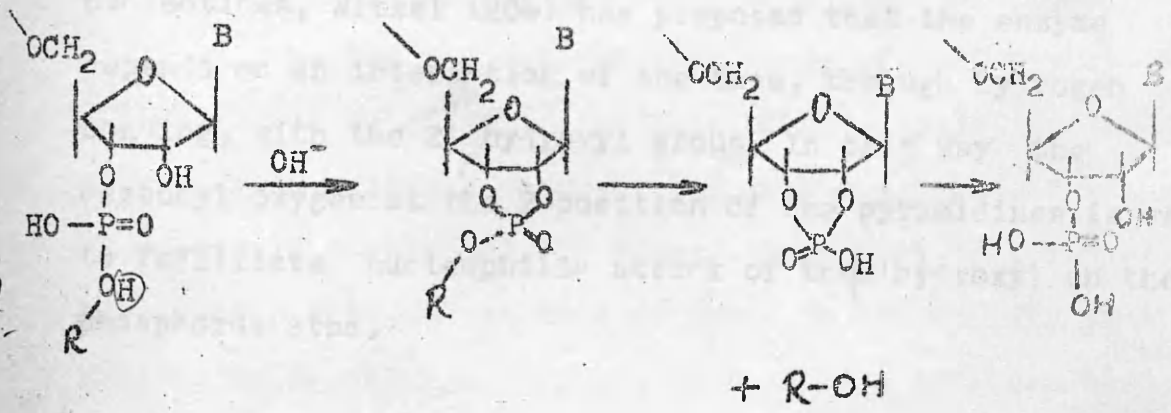
greater rate of destruction of R3P over that of ribose in this way and Baron and Brown (199) have proposed that decomposition of R3P proceeds via a $\beta$-elimination:

This reaction requires an electron withdrawing group on the carbon atom adjacent to the one bearing the group which is eliminated. This is the case for the 3-phosphate group (which is $\beta$ to the 2 carbon atom which bears the aldehyde group) but not for the 2-or 5-phosphates. This course of destruction cannot be followed by ribose, in which there is no phosphate group.

The results of Khym et al. are best accommodated by combining the two hypotheses. Thus, destruction of ribose and R5P proceeds by oxidation of the 2,3-and 3,4-ene-diols; destruction of R3P by oxidation of the 2,3-ene-diol is accompanied by a $\beta$-elimination. R2P is stable because neither course of destruction is available to it.

C The hydrolysis of RNA by alkali and ribonuclease.

Brown and Todd ( 17 ) proposed that alkaline hydrolysis of RNA involves an initial formation of a 2':3' cyclic-5'-phosphotriester:

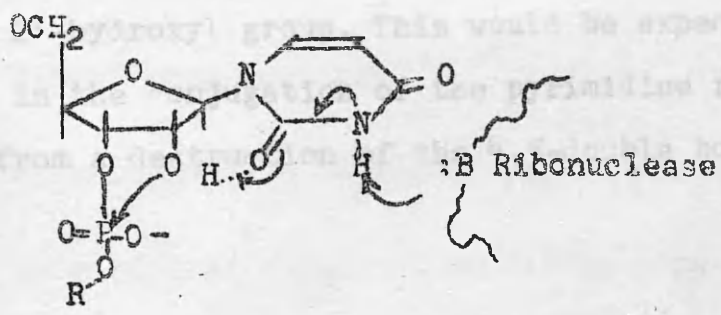



This mechanism explains the formation of mixed 2'-and3'-phosphates from the 3'-5'-phosphodiester linkages and also the greater stability to alkali of DNA, in which 2':3' cyclic phosphate formation is not possible.

A similar mechanism was proposed ( 17 ) for the action of pancreatic ribonuclease. In this case, the cyclic phosphate formed initially is split specifically at the 2'-linkage, to give the 3'-phosphate. It has been shown that this similarity between ribonuclease and alkaline hydrolysis of RNA can be extended. It has been known for some time

that RNase attacks only those phosphodiester linkages in RNA which are adjacent to pyrimidine bases. Recent experiments (205) show that these bonds are also more labile to alkaline hydrolysis than those adjacent to purines.

To account for the specifity of RNase to pyrimidine nucleotides, Witzel (204) has proposed that the enzyme depends on an interaction of the base, through hydrogen bonding, with the 2' hydroxyl group. In this way the carbonyl oxygen at the 2-position of the pyrimidines is seen to facilitate nucleophilic attack of the 2'hydroxyl on the phosphorus atom.




This proposal is in keeping with the observed specificity of ribonuclease. Those compounds which are able to form hydrogen bonds to the 2'-hydroxyl group e.g. derivatives of pseudouridine (208), thiouridine(208) dihydrouridine(204, and SectionIII of this thesis) and polyribosephosphate (145) are hydrolysed by the enzyme. The enzyme fails to hydrolyse

purine derivatives and methylribofuranoside 2:3-cyclic phosphat
20[7]) in which hydrogen bonding between the aglycone and the 2'-hydroxyl group is impossible.

Witzel reported (204) that RNase is able to hydrolyse dihydrouridine-3'-phosphodiester linkages, but did not make observations on the reaction rate.

The reduced rate of hydrolysis of polydihydrouridylic acid relative to the rate of hydrolysis of polyuridylic acid was demonstrated in the experiment discussed in SectionIII. It seems likely that this reduction in reaction rate is due to a decrease in the ability of the pyrimidine 2-carbonyl oxygen atom to form a hydrogen bond with the 2'-hydroxyl group. This would be expected from the decrease in the conjugation of the pyrimidine ring which results from a destruction of the 4,5-double bond.

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## General methods and procedures.

(abbreviated G.M.)

### G.M. 1. Determination of pentose containing compounds.

The method of Mejbaum (210) was used.

The reagent prepared by dissolving orcinol (recrystallised from benzene) in a stock solution of ferric chloride (0.1% in conc. HCl) to a final concentration of 1.0%. This solution was discarded after one day.

The sample, containing from 5 to 40µg of pentose was diluted to 4ml with water and mixed with the reagent (4ml) in a rimless test-tube (⅜" x 6"), which was covered with a glass marble and placed in a boiling water-bath for 40min. The colour developed was measured in the E.E.L. colorimeter (filter 608; 660mµ) when the solution had cooled to room temperature.

Pentose content was estimated by comparing the colour with a standard curve prepared by treating

known amounts of ribose as above.

G.M. 2. Determination of inorganic acid-labile, and ester phosphate.

The method of King (209) was used

Three solutions were required:

A. 60% perchloric acid (A.R.).

B. 5% ammonium molybdate (A.R.).

C. 0.5g 1-amino-2-naptholsulphonic acid (amidol), 30g anhydrous sodium bisulphite made to 250ml with distilled water and filtered. This solution was discarded after one week.

Inorganic phosphate.

The sample, containing 10-100µg of phosphorus was mixed with solution B (1ml) and solution C (0.5ml) in a test tube (½" x 6") graduated at 15ml. The mixture was diluted with distilled water and solution A (1.2ml) added. The volume was made to 15ml. After mixing, the

difference that no further perchloric acid was required.

For both acid-labile and ester phosphate, a "blank" determination of inorganic phosphate was always carried out on a similar volume of the sample.

G.M. 3. Determination of reducing sugars by the Somogyi-Nelson procedure.

Copper Reagent A. Anh. sodium carbonate (40g), Rochelle salt (25g) and anh. sodium sulphate (20g) were dissolved in water (800ml) the solution was filtered and made up to 1l. The solution was stored at 30°C.

Copper reagent B. Copper sulphate pentahydrate (15g) in water (100ml) was acidified with one drop of conc. sulphuric acid.

Arseno-molybdate reagent. Ammonium molybdate (25g) was dissolved in distilled water (450ml) and conc. sulphuric acid (21ml) added. To this was added a solution of disodium hydrogen arsenate (3g.) in water (25ml) and the mixture kept at 37°C for 2 days. The solution was stored in brown bottles.

The sample containing reducing sugar (1ml containing the equivalent of 10-50µg ribose was mixed with the alkaline copper reagent (1ml of a mixture of 25ml soln. A + 1ml soln. B) in a test-tube (½" x 6"), which was covered with a glass marble and placed in a boiling water-bath for 30min. After cooling to room temperature, the arseno-molybdate reagent (1ml) was added and the solution made up to 15ml with water. The colour produced was measured in the E.E.L. colorimeter (filter 608, 660mµ) against a water blank and compared with a calibration curve obtained by treating standard solutions of ribose in the same manner.

G.M. 4. Modification of colorimetric procedures to a smaller scale.

Frequently, it was necessary to determine amounts of material smaller than the limit of sensitivity of G.M. 1-3. In these cases, the scale of these procedures was reduced by reducing the volumes of all the reagents proportionally. A final volume of 1ml was usually chosen. The colours were measured in 1cm glass cuvettes using the Unicam SP 400 spectrophotometer at 660mµ against a

water blank. The cuvette was supported so that the whole of the light area passed through the solution.

Despite the necessity of using less accurate micropipettes, the accuracy achieved by these modifications was at least as high as the standard procedure.

G.M. 5. Paper chromatography.

For descending development, samples were applied at intervals of 2cm along a line 3" from one edge of the filter paper. The paper was clamped between three glass rods which were supported in such a way that the end of the paper dipped into a trough of the developing solvent. Development was carried out in accumulator jars, sealed with a glass plate over a thin sheet of polythene. When it was necessary to prolong development beyond the lower end of the paper, this edge was serrated.

For ascending development, the samples were applied 1" from the lower edge of the filter-paper. The paper was held, during development at three points along each vertical edge, in a frame of "Teflon" so

that the lower edge of the paper rested in the solvent.

The following solvents were used:

Solvent 1. isopropanol /1% aqueous ammonium sulphate (2:1)

Solvent 2. isopropanol/1% aqueous ammonium borate (2:1)

Solvent 3. 95%ethanol/1M ammonium acetate pH 3.5 (5:2)

solvent 4. 95%ethanol/1M ammonium acetate pH 7.5 (5:2)

Solvent 5. water containing ammonia (pH 10)

Solvent 6. t-butanol/2% aqueous picric acid (8:2)

Solvent 7. n-butanol/acetic acid/water (74:19:50)

G.M. 6. Electrophoresis on filter-paper strips.

The sample was applied carefully to a point on a narrow filter-paper strip, so that the area covered by the solution was approx. 5mm in diameter. The starting line was 10cm from one end of the paper strip.

The "long" end of the strip was dipped through the buffer solution to be used up to a point approx. 1cm from the starting line and allowed to approach the starting line by capillary action. Excess buffer was blotted off with dry filter paper and the process was repeated on the "short" end of the strip. In this way excessive diffusion of the spots was avoided. The central portion of the paper was hung in an accumulator jar containing carbon tetrachloride. The ends were dipped into two similar jars containing the conducting buffers and the electrodes. Current was passed for a prescribed period after which the paper was dried at 110°C.

Buffer solutions used were:

Buffer 1. water/pyridine/acetic acid pH 6 (945:50:5)

Buffer 2. 0.05M borax pH 9.2

G.M. 7. Detection of compounds on filter paper.

1. Reducing sugars.

a. alkaline silver nitrate

Reagents required were:

i. Silver nitrate (0.5ml satd. soln. in water)

was diluted with A.R. acetone (100ml) and water was added until the precipitate just dissolved.

ii. Caustic soda (2g) was dissolved in water (5ml) and made to 100ml with ethanol.

The paper was dipped rapidly through reagent i, allowed to dry in air and sprayed lightly on both sides with reagent ii. The spots were allowed to develop until maximum contrast was obtained. The excess silver salts were then washed out by immersing the paper in aqueous ammonia (6N) after which it was washed in running water for 1hr.

b. Benzidine/TCA

Benzidine (0.5g) was dissolved in glacial acetic acid (10ml) and the solution mixed with trichloracetic acid (10ml, 40% w/v) and ethanol (80ml). The reagent was sprayed lightly onto both sides of the paper which was then heated at 85°C until the spots reached maximum intensity.

## 2. Phosphate esters.

Molybdate-perchloric acid reagent

The paper was sprayed with a solution of ammonium molybdate (1% w/v) in perchloric acid (3%) and dried for 1min at 85°C. The phosphate esters formed blue spots, on exposing the paper to u.v. radiation.

## 3. Amino-derivatives including uridines.

Ehrlich's reagent.

The paper was dipped through a solution of p-dimethylaminobenzaldehyde in acetone (1% w/v) containing hydrochloric acid (3ml% conc. HCl). Intense yellow colours were given by $-NH_2$ compounds after hanging in air for several hours.

G.M. 8. Preparation of polyphosphoric acid for phosphorylations

Orthophosphoric acid (A.R., 85% w/v) was heated in a beaker to approx. 60°C and phosphorus pentoxide was added slowly, with stirring to avoid formation of lumps (the proportions of $H_3PO_4$ and $P_2O_5$ were 1.3:1 w/w).

when solution was complete, the mixture was cooled to 60°C before adding the substance to be phosphorylated.

G.M. 9. Ion-exchange chromatography.

a. Phosphate esters.

Phosphate esters were fractionated on Dowex 1 x 8 (200-400 mesh). The resin was pretreated by suspending in a large volume of water and decanting the "fines" which failed to settle in 15min. This process was repeated using three alternate cycles of 0.5 NaOH and 1N HCl with an aqueous wash between each treatment. After the last acid treatment the resin was washed with water until the washings were approx. pH 5 and poured as a dilute slurry into a glass column of suitable dimensions. Small columns (1cm i.d.) had a sintered glass disc to hold the resin. Larger columns were packed at the lower end with a layer of glass-wool covered by a pad of cotton-wool.

The resin was washed in the column by passing a. potassium hydroxide (N/2 approx., 50 column volumes)

b. water to neutrality c. hydrochloric acid (2N, approximately 50 column volumes) d. water to neutrality.

Phosphate esters were applied to the column as sodium or ammonium salts ( pH 6.0) in dilute solution ($\mu = 0.05$).

b. Nucleosides.

The resins used in chromatography of the nucleosides were Amberlite IR 120 and Zeokarb 215. These were passed through washing cycles similar to those used with Dowex 1 and columns were prepared as above.

Elution of columns was carried out in the cold-room for the phosphate esters. Details of eluents used are given in the appropriate experimental section. Linear increase of eluent concentration was achieved by feeding a solution of the highest required concentration into water or a solution of low ionic strength in the mixing chamber. The two solutions were contained in identical conical flasks. The reservoir had a single outlet at its base which was connected to an inlet at the base of the mixing chamber. The solution in the

mixing chamber was stirred mechanically and withdrawn to the column from an outlet at the base.

G.M. 10. Growth of E. coli

The liquid culture medium contained yeast extract (1% w/v; Difco, dehydrated), glucose (1%), dipotassium hydrogen phosphate (2.18%), monopotassium dihydrogen phosphate (1.7%). Glucose was prepared in a separate solution and mixed with the other constituents after autoclaving (15lb.in$^{-2}$, 20 min).

A freeze-dried preparation of E. coli strain B was used to inoculate approximately 2ml of the growth medium which was then incubated at 37°C overnight. Samples withdrawn from the resultant cell suspension were used to seed nutrient agar slopes which, after overnight incubation at 37°C, were stored, sealed, at 4°C.

Inocula for large cultures were prepared in the following way. A scraping of cells from an agar slope was transferred by means of a sterile platinum loop to the liquid culture medium (3ml) in a sterile

test-tube plugged with cotton-wool. This was incubated (37°C) overnight and a drop of the resultant cell suspension transferred, aseptically, to a similar test-tube containing fresh growth medium. This cycle was repeated twice and the contents of the third test-tube were used, after incubation, to inoculate 1.5l. of the growth medium in a 5l. flask. This was cultivated at 37°C overnight with vigorous aeration and used to inoculate 7.5l. of the culture medium contained in a 10l. bottle. The large cultures were grown at 37°C with vigorous aeration and the cells harvested at room temperature after 3-4 hr cultivation, in a Sharples or M.S.E. continuous-flow centrifuge.

G.M. 11. Assay of polynucleotide phosphorylase.

The stock solution was Tris buffer pH 8.2 (0.2M) containing magnesium chloride (0.01M). This solution was stored over chloroform. ADP (97mg, commercial sodium salt) was dissolved in the stock solution (10ml). The ADP solution was stored at 4°C and discarded after a few weeks.

For assay, this solution (0.1ml) was mixed with the enzyme solution (0.1ml) in a conical centrifuge tube and digested at 37°C (digestion time was 5, 15, 30 or 60 min according to the concentration of enzyme). Reaction was stopped by the addition of perchloric acid. (5.75ml, 6%). The precipitate of protein was removed by centrifugation or by filtering through a glass-wool plug according to its physical form. Molybdate solution (0.5ml) and amidol reagent (0.25ml) were added and the blue colour measured as described in G.M. 2. A blank test in which the perchloric acid was added before the enzyme was always carried out and the phosphate concentration subtracted from the test value.

A unit of activity was that amount of enzyme which would release 1 micromole of inorganic phosphorus per hr under the conditions of the assay. Specific activity was units of activity per milligram of protein.

G.M. 12. Measurement of protein concentration.

a. The biuret test.

The reagent was prepared by stirring a solution of sodium hydroxide (150ml, 10%) into a solution containing copper sulphate penta-hydrate (0.75g) and Rochelle salt (3g). The volume was made to 500ml with water and the reagent stored in polythene bottles.

The sample (1ml) containing 1 to 10mg of protein was mixed with the reagent (4ml) and allowed to stand at room temperature for 30min. The colour produced was measured in the E.E.L. colorimeter (filter 605, 550mμ) and compared with a standard graph. The method was calibrated using solutions of bovine serum albumin of known concentration.

b. Folin-Ciocalteau reagent.

The following reagents are required:

Reagent A. 2% $Na_2CO_3$ in 0.1N NaOH

Reagent B. 0.5% copper sulphate penta-hydrate in 1% sodium tartrate.

Reagent C. 50ml of reagent A are mixed with 1ml of reagent B. This solution is kept for no longer than one day.

Reagent E. Sodium tungstate (100g), sodium molybdate (25g), phosphoric acid (50ml, 85%), conc. hydrochloric acid (100)ml and water (700ml) were refluxed for 10hr. The solution was filtered and diluted to 1l. The concentration of acid was determined and adjusted to 1N by dilution.

Test was carried out by mixing the sample (1ml) with reagent C (5ml) and allowing the mixture to stand at room temperature for 30min. Reagent E (0.5ml) was added with shaking, and after 10min the blue colour was read in the E.E.L. colorimeter (filter 608, 660mμ). Bovine

serum albumin was used to prepare the standard curve.

G.M.13. Preparation of a standard bentonite suspension.

A suspension of bentonite (10g, British Drug Houses) in water (200ml) was allowed to settle for 15min. The fine suspension was decanted from the coarse particles, which were discarded , and centrifuged (9000g for 20min.). The supernatant suspension was discarded . The sediment was resuspended in water and the cycle repeated until no clay remained in suspension after 20min. at 9000g. Finally the bentonite was suspended in water (50ml). The suspension was thoroughly shaken before withdrawing samples in a pipette.

G.M.14 Estimation of ribonuclease activity.

A stock solution containing purified commercial yeast RNA (L. Light and Co. Ltd.) was prepared in the Buffer used for polynucleotide phosphorylase assay (G.M.11) containing 100mg. of RNA in 10 ml of buffer. To this solution (0.2ml) was added enzyme solution (0.2ml) and the mixture was incubated at 37°C for 30min. Trichloracetic acid (0.4ml) 20%w/v) was added and the precipate removed in the centrifuge. An aliquot (0.2ml) of the supernatant solution was taken for total phosphate estimation (GM2).

A blank test in which the RNA solution was mixed with the trichloracetic before the addition of the enzyme, was always carried out.

The method devised for measuring the activity of ribonuclease on acid soluble polymers is described in G.M.19.

G.M.15 Preparation of Norit A for adsorption of RNA.

The use of Norit A in adsorption of RNA has been described Zamenhof and Chargaff (144) Norit A was washed overnight by gently running tap water in a tall cylinder. The suspension was then centrifuged (20mins. at 3,000xg) and washed with 2M sodium chloride solution (8x) and then 0.14M sodium chloride solution (2x). Each washing was followed by centrifuging as above. Finally the charcoal was suspended in sufficient 0.14M sodium chloride to give a thick slurry.

GM16 Preparation of Calcium Phosphate gel.

The method of Tsuboi and Hudson was used (187).

Disodium hydrogenphosphate (14.2g) was dissolved in water (200ml) and conc. ammonia (6ml) added. This was

followed immediately by the addition of a solution of calciumchloride(32.9g of the hexahydrate in 1.5l). The suspension was stirred thoroughly and adjusted to p 7.8 by addition of hydrochloric acid. The gel was centrifuged down(20min. at 1,500g) and washed several times with distilled water.

G.M.17. Preparation of Alumina C8 gel.

A solution of aluminium ammonium sulphate(340g) in water (500ml.) was poured rapidly with vigorous stirring into a solution containing ammonium sulphate (100g) in a final volume of 3.25l. Stirring was continued for 15mins. When the precipitate became flocculent,water(17l) was added and the precipitate allowed to settle.The aqueous wash was repeated twice. During the fourth wash ammonia(40ml,20%) was added. Washing with water was continued until the supernatant solution would not clear,after which the gel was washed twice more. Finally the gel was suspended in

sufficient water to give a concentration of 20mg dry weight per ml.

G.M.18. Gel Filtration on Sephadex columns.

Dry Sephadex(G25 fine grade) was stirred with the appropriate salt solution(0.2MNaCl on $NH_4HCO_3$ were used) to swell the gel grains, for 1-2hr. The column to be used was attached by a piece of wide tubing to a funnel and was filled with the salt solution before adding the gel. The suspension of gel in the funnel was stirred mechanically and allowed to settle through the solution in the column until a layer approximately 1" thick had formed. Buffer flow was now commenced at such a rate that a sharp horizontal surface was always discernible on the gel bed.

When all the gel was settled washing with salt solution was continued until approximately 50 bed volumes had passed.

For sample application, the salt solution was allowed to drain until the gel surface was just dry. The solution to be fractionated was applied carefully by gently pipetting down the side of the column from a height over 3" above the gel surface. The sample(always contained in less than

0.1 bed volumes) was allowed to drain into the gel and elution was continued with the salt solution. First applications of the salt solution were made with a pipette in the same manner as described for the sample.

G.M.19 Fractionation of polyribotides on ECTEOLA cellulose. ✓

The cellulose(Whatman powder ET 30) was washed by suspending successively in a 0.5M sodium hydroxide b) distilled water c) 0.1M hydrochloric acid and d) distilled water This washing cycle was repeated three times(prolonged contact of the cellulose with the acid was avoided). After the final acid wash the cellulose was washed with water until the washings were neutral. Columns were prepared by pouring a thick slurry of the cellulose ,in water, to the required height. The sample to be fractionated was applied to the column in dilute solution($\mu < 0.1$) at a fairly fast flow rate(approximately 0.1 bed volumes per minute). After a short wash with water the low molecular weight ... material was eluted with 0.5M lithium chloride(at least 10 bed volumes

Higher molecular weight material was then eluted with a similar volume of 0.25M lithium chloride solution.

For the assay of ribonuclease described at the end of section III, the ECTEOLA cellulose "column" consisted of a small plug(approx.3mmx3mm.) at the end of a glass tube which was stopped by a glass bead. To facilitate collection of solutions, the tube was held on the rim of a test -tube by a substantial ribbon collar, surrounding the column about 2cm from its lower end. After application of the sample and a water wash, the cellulose was eluted twice with 0.05M lithium chloride(1.0ml and 0.5ml) and the effluents made up to 3ml.

G.M.20. Preparation of dibenzylphosphite and dibenzyl-phosphorochloridate.

The preparation of dibenzylphosphite was carried out as described by Friedman et al.(166)

A mixture of dry pyridine(158ml) was added slowly (3hr.) with stirring to a mixture of phosphorus trichloride(88ml) in dry benzene(75ml0 The reaction flask was cooled in ice. After all the mixture had been added stirring was continued for 30min., and then a further amount(104ml) of benzyl alcohol was added. The mixture was

allowed to stand at room temperature for 16hr, after which it was washed successively with water(3x500ml), 5Nammonia (2x500ml), and water(2x500ml). After drying the solution over anh.sodium sulphate, benzene was removed at room temperature under reduced pressure and benzyl chloride on the steam bath at 1-3mmHg.

Conversion of dibenzyl phosphite to dibenzyl phosphorochloridate was effected by mixing equimolar quantities of dibenzylphosphite and N-chlorosuccinimide, in benzene solution. After 2hr. at room temperature the succinimide was filtered off(through a glass filter) and the benzene solution of dibenzylphosphorochloridate was either used direct ,or the solvent was removed under reduced pressure and the resultant oil dissolved in dry pyridine.

G.M.21. Chromatography on DEAE-cellulose.

The cellulose (whatman,DE30) was put through a washing cycle similar to the one used with ECTEOLA-cellulose(G.M.19) and columns were prepared in a similar manner. Protein solutions were dialysed before application

to the column. All operations subsequent to preparation of the column were carried out in the cold room at 4°C. Flow rate was always less than one column volume per hour.

G.M.22. Interconversion of salts of phosphate esters.

a) Conversion to acidic form.

The metal salt($Ba^{++}$, $Ca^{++}$, $Na^{+}$, $Li^{+}$) was dissolved in water (approx 10mg/ml) if necessary with the addition of a small amount of ion exchange resin(Amberlite IR120 $H^{+}$) or acetic acid. The solution was then applied to a column of the same resin(approx,6equivalent) which was washed with distiled water until the effluent was no longer acidic. The acid form was either stored in frozen solution or freeze-dried.

b) Conversion to other forms.

The salt was first converted to the acidic form as described above and brought to neutrality using the appropriate base.

G.M. 23. Estimation of uncombined glycosidic positions in pentose compounds by reaction with sodium borohydride.

The sample is dissolved in Tris buffer (ph7.4,0.2M) so that the concentration of pentose is approximately 25mg/ml. Two aliquots of this solution (0.2ml) was taken and to one of them sodium borohydride (4mg) was added. After standing at room temperature overnight, the pentose in both solutions is measured by the ferric-orcinol method (G.M.1). The amount of pentose reduced by sodium borohydride is the difference between these two figures, corrected as described below.

A pentose compound which is fully uncombined in the glycosidic position produces a colour with the ferric-orcinol reagent after treatment with sodium borohydride, and since this colour shows some absorption at 660mμ, it is necessary to take the "residual colour" into account in calculating the number of free glycosidic positions from its orcinol figures obtained with a test compound.

If it is assumed that the "residual colour" produced by each compound is the same, necessary correction

can be calculated in the following way.

Two series of solutions containing increasing quantities of a pentose standard (R5P was used in this work) are prepared in Tris buffer (0.2ml,0.2M,pH7.4). To one series,sodium borohydride (4mg for each sample) is added and the solutions are allowed to stand overnight at room temperature. Orcinol tests are carried out on each series and the "pentose" values calculated from these figures.The "residual colour" pentose values are now subtracted from the total pentose values and the "residual colour" values plotted against the difference.

To find the correction to be applied to a test compound,the difference between the total pentose and pentose after sodium borohydride treatment is measured as described above and the correction for "residual colour" is read from the curve obtained with the standard pentose compound. The correct value of the uncombined pentose is the sum of these two figures. The value is most conveniently expressed as a percentage of total pentose.

## Experiments for Section I.

### Expt. 1. Preparation of 1-O-methyl2:3-O-isopropylidene-D-ribofuranose.

D-Ribose (20g.) which had been dried over $P_2O_5$ (18 hrs., 90°C, $10^{-1}$ mm) was stirred for 24 hrs. with a mixture of acetone (380 ml), methanol (20 ml), conc. sulphuric acid (0.8ml), and anhydrous copper sulphate (40g.) in a flask from which the atmosphere was excluded. The copper sulphate was removed by filtration and the sulphuric acid by shaking for 2 hrs. with an excess of anhydrous calcium hydroxide. After filtration the solvents were removed at room temperature under reduced pressure. The residual light yellow syrup was distilled (80°C, 0.01mm Hg). The distillate, a colourless syrup, weighed 18.8g (70.6% of theory). Microanalysis: C = 52.38%; H = 7.57% (theory C = 52.94%, H = 7.84%). Pentose (G.M. 1) = 71.0%, (theory = 73.9%).

### Expt. 2 Synthesis of ribose 5-phosphate using dibenzylphosphorochloridate.

A solution of 1-O-methyl-2:3-O-isopropylidene

-ribofuranose (4.5g) in anhydrous pyridine (50ml) was cooled to -40°C. and a solution of dibenzylphosphoro-chloridate (from 16 g dibenzylphosphite, in 164ml carbon tetrachloride at -10°C). The mixture was kept at -40°C for 3hrs and then at room temperature for a further 3 hrs. Sodium carbonate (7g of anhydrous) and water (30ml) were added and solvents removed at the oil-pump using a 25°C water-bath. The solid residue was extracted with chloroform (2 x 40ml) and the leached solid was discarded. The solution in chloroform was washed with saturated aqueous sodium bicarbonate (3 x 50ml) and distilled water (2 x 50ml), and then dried over anhydrous sodium sulphate (18hrs). A mobile, red oil (6.5g) was obtained by removing the chloroform under reduced pressure and this was dissolved in 50% aqueous alcohol (100ml). Acetic acid (1ml, 5N) and Adam's platinum catalyst (10mg) were added. Hydrogenation at room temperature and atmospheric pressure was judged complete after 5 hrs when 580cc had been consumed (theory = 625cc). The catalyst was filtered off and the filtrate reduced to 10mls by evaporation at the water- pump. The volume was made to 400ml by the addition of N/3 sulphuric acid and this solution was

left at room temperature for 18 hrs, after which it was brought to pH 8.0 by the addition of barium carbonate and saturated barium hydroxide solution. The insoluble barium salts were removed by filtration and washed on the filter pad. Filtrate and washings were combined and evaporated to 40cc under reduced pressure. A slight precipitate which appeared during evaporation was centrifuged off. The supernatant solution was poured slowly into absolute alcohol (200ml) and the flocculent white precipitate which formed was centrifuged down, successively washed with alcohol and ether and dried in vacuo. The yield was 820mg (10.2% of theory).

Analysis: pentose = 10.1% (theory based on formula weight $BaR5P$ = 365 is 41.0%).

Expt. 3. Repeat of Expt. 2 with pentose analysis at each step.

The procedure of Expt. 2 was followed exactly using double the quantities of all reagents and solvents. At each stage of the reaction a suitable aliquot of the solution was taken and diluted. Portions of this solution were analysed for pentose (G.M. 1) and, after the

hydrogenolysis of benzyl groups, for inorganic ortho-phosphate (G.M. 2). The results are shown in Table 2. The total uptake of hydrogen in this experiment was approximately 41. The final addition of ethanol to the barium salts caused no precipitation.

Expt. 4. Synthesis of ribose 5-phosphate using a purified dibenzyl phosphorchloridate.

In this preparation 1-O-methyl-2:3-O-isopropylideneribofuranose (0.45g) was reacted with dibenzyl phosphorochloridate synthesised by a new method (G.M. 20). The phosphorylating agent, as well as the substrate, was dissolved in pyridine. After reaction, which was carried out as previously (Expts. 2 and 3) pyridine was removed by repeated evaporation of a solution in chloroform. Hydrogenation was rapid and smooth (30 min, 96 cc. theory = 62.5cc) over palladium chloride (200 mg). After hydrogenolysis of the benzyl groups, a gum was obtained by freeze-drying. This was dissolved in ethanol (14 ml) and poured into acetone, a stiff white gum precipitated which weighed 0.39g after removing the acetone by decantation and evaporation. The blocking

groups on the sugar were removed as previously and the Ba R5P isolated in the same way. The yield at each stage is given in Table 5.

Expt. 5. Preparation of ribose-5-phosphate using dibenzyl phosphorochloridate; modified procedure.

The reaction was carried out in exactly the same manner as Expt. 4, using dibenzyl phosphorochloridate (from 5g purified dibenzyl phosphite) and 1-O-methyl-2:3-O-isopropylideneribofuranose (1.5g). Similarly the P,P-dibenzyl-1-O-methyl2:3-O-isopropylideneribofuranose-5-phosphate was isolated after reaction. At this stage the oil was dissolved in ethanol (20cc, 50%aqueous) and treated with Dowex 2($Cl^-$)(1g) and then with Amberlite IR 120 ($H^+$)(1g). The resin was shaken with the solution for 20 mins and then filtered off. A test for chloride at this stage was negative and the pH of the solution was 6.3. Quantitative pentose analysis (G.M. 1) gave a value for pentose = 90% theory. After hydrogenation, paper electrophoresis (Solvent 1, G.M. 6) showed that the product at this stage contained only a trace of inorganic phosphate. The methyl and isopropylidene groups were

removed as in Expt. 4. After 18 hrs in N/3 sulphuric acid the pH was adjusted to 8.2 by the addition of 5N potassium hydroxide and aqueous barium acetate (25ml of 25%) was added. The precipitate was removed in the centrifuge and 4 volumes of absolute alcohol were added to the supernatant solution. Precipitation was completed by keeping the solution in the refrigerator overnight. A second crop of Ba R5P was obtained by digesting the first barium precipitate (formed on addition of barium acetate) with 0.1N hydrochloric acid at room temperature for 2 hrs, neutralising as before and treating the solution with 4 volumes of alcohol. The yield at each stage in this preparation is shown in Table 6. The pentose analysis of the two barium salt fractions showed them to be 53% and 70% pure (1st and2nd crops respectively). On this basis the final overall yield from the two fractions is 30.3%.

Expt. 6. Preparation of R5P using polyphosphoric acid: determination of optimal reaction time by colorimetric analysis.

1-O-methyl-2:3-O-isopropylideneribo

furanose (150mg) was dissolved in freshly prepared polyphosphoric acid (1ml, 1/1.3 w/w) (G.M. 8). The mixture was maintained at 60°C in a thermostatically controlled water-bath. Reaction was carried out in a sealed flask and the mixture was stirred at intervals. The mixture became dark brown after approximately 10min. Aliquots (0.2ml) were removed at ½hr intervals and diluted with water (1.8ml). Because of the viscous nature of the liquid, the pipette in which the aliquots were withdrawn, were washed with the solution by repeatedly sucking it into the pipette. After 3 hrs, when 5 withdrawals had been made, the diluted solutions were heated in a boiling water-bath for ½hr and while still hot the pH was adjusted to 6.5 by the addition of baryta. Each solution was filtered and made up to 100ml with water. Samples of these diluted solutions were taken for pentose (G.M. 1) and inorganic and total phosphorus estimation(G.M. 2). The results (Table 3) are illustrated in Fig. 4.

Expt. 7. Preparation of R5P using polyphosphoric acid: determination of optimal reaction time by paper chromatography.

Into separate round-bottomed flasks

were weighed ribose (190mg) and 1-O-methyl-2:3-O-isopropylideneribose (250mg). To each was added freshly prepared polyphosphoric acid (G.M. 8) (1.6ml) and this was mixed thoroughly with the sugar. The flasks were sealed and kept at 60°C in a thermostatically controlled water-bath. At ½hr intervals aliquots (0.2ml) were withdrawn and treated exactly as described in Expt. 6. The precipitate of barium phosphate, in this case, was centrifuged down, and without further treatment the supernatant solution was spotted on Whatman's No. 1 chromatography paper. Ten applications of each solution, were applied at a spot on the origin line. The chromatograms were developed ascendingwise for 21 hrs in Solvent 1, and the spots detected by reagent 1 (G.M. 7). The intensities of the spots were compared visually.

Expt. 8. Preparation of R5P using polyphosphoric acid (1).

Dry ribose (5g) was mixed with polyphosphoric acid (42ml) and the mixture kept at 60°C with the exclusion of moisture for 1½ hrs. The ribose was not quite completely dissolved after this time. Water (200ml) was added and the solution heated for ½hr on the steam

bath. The solution, which was red-brown, was filtered from charred material and stirred with an excess of barium carbonate until evolution of $CO_2$ ceased. Solids were removed by filtration and the filter cake washed with hot water (100ml). The filtrates were combined, heated to near boiling and adjusted to pH 6.5 with hot saturated baryta. The precipitate was filtered off. The volume of the filtrate at this stage was 1½l. and this was reduced to 50ml (at 70°C, under reduced pressure). More precipitate fell during evaporation and this was filtered off and washed with a small amount of hot water. Filtrate and washings were combined, adjusted to pH 8.2 with saturated baryta and filtered. The addition of 4 volumes of cold ethanol to the filtrate caused a flocculent precipitate to form, and this was centrifuged down. After washing with ethanol and ether, the precipitate was dried in vacuo (0.92g). The yield was 10% of theory and, on the basis of pentose, contained 80% $BaR5P$.

Expt. 9. Preparation of R5P using polyphosphoric acid (2).

This was carried out as Expt. 8 using 20g of dry ribose and proportionally more polyphosphoric

acid. Before precipitating the Ba R5P with ethanol, the solution was decolourised by boiling with activated charcoal (B.D.H.) and filtered. The yield of the barium salt was 2.5g (6% of theory).

Expt. 10. Preparation of uridine from yeast nucleic acid.

Commercial yeast nucleic acid (100g) was dissolved in 50% aqueous pyridine (800 ml) in a 5l. round bottomed flask fitted with a reflux condenser. The solution, which was dark brown in colour, was brought to boiling on an electric mantle and kept at boiling point for 100 hrs. The severe bumping was lessened by frequent addition of small chips of porous pot. After the hydrolysis period, solvents were removed in the rotary evaporator at 70°C, using an oil-pump and liquid air trap to speed evaporation. The dark residue was dissolved in water (200ml) and boiled with activated charcoal (15g, British Drug Houses). The decolorised solution was filtered while still hot and reduced in volume(150ml). Guanosine was crystallised by standing the solution in the refrigerator overnight and was removed in the centrifuge (7.2g). The supernatant solution was taken

to pH 8.2 with saturated aqueous baryta and the precipitate of barium phosphate removed by filtration. The filtrate was reduced to 80ml, 400 ml of absolute alcohol was added, and the precipitate filtered off. A gum was obtained by removing the solvents and this was dissolved in water (1.5l.).

20ml of this solution was applied to a column (1.0cm x 14cm) of Amberlite IR 120 ($NH_4^+$), which was eluted with water (200ml) and then $NH_4OAc$ (0.1M, pH 9.0). Fractions (10ml) were collected and $O.D._{260}$ was determined. Results are illustrated in Fig. 5. The peaks were concentrated by evaporation and paper chromatography (Solvent 5) used to identify their contents.

The rest of the solution was applied to a large column (3.5cm x 30cm) of Zeo-karb 215 ($H^+$). The effluent was collected in 200ml fractions and a sample of each was applied at the origin of a paper chromatogram. The column was eluted with water (1.5l.) until the spots on the paper could not be detected under a U.V. lamp. The fractions eluted with water were bulked

(Peak I). The basic nucleosides and adenine were eluted together by aqueous ammonia (0.1 N). Fractions were tested for U.V. absorbing material as above. All those containing U.V. absorbing substances were bulked (Peak II). Development of the paper chromatogram (Solvent 5) showed that fractions eluted with water contained only uridine. Those eluted with ammonia contained adenosine and adenine. The eluate containing peak I was reduced in the rotary evaporator, last traces of water were removed by azeotropic distillation with absolute alcohol. The product was a light yellow solid (16g). Crystallisation from 95% ethanol yielded 5g of white crystalline uridine. The rest (11g) was recovered from the mother liquors as a light yellow, amorphous solid by evaporating the ethanol.

Expt. 11. Preparation of 2':3'-O-isopropylideneuridine.

Uridine (5g of the amorphous fraction, Expt. 10), dried over $P_2O_5$ at 55°C and $10^{-1}$ mm Hg, was mixed with anhydrous copper sulphate (10g), dry acetone (125ml) and concentrated sulphuric acid (0.125ml) in a sealed flask fitted with a mechanical stirrer. The flask was held

in a thermostatically controlled water-bath at 37°C for 72 hrs and throughout this period, the mixture was vigourously stirred. The copper sulphate was removed, at the end of the reaction, by filtering the solution and the sulphuric acid was removed by shaking the filtrate with calcium hydroxide (5g) for 1hr. The calcium salts were filtered off and the solvents removed from the filtrate by evaporation under reduced pressure. The yellow gum which resulted was dissolved in hot methanol and allowed to crystallise (4.0g, 65% theory).

A similar experiment with double the quantities of all reactants produced 11g of isopropylidene uridine on evaporation of the acetone. This was a clear white solid and was not crystallised from methanol.

Expt. 12. Preparation of uridine-5'-phosphate: determination of optimal reaction time.

2':3'-O-Isopropylideneuridine (200mg) was mixed thoroughly with polyphosphoric acid (1ml) in a stoppered flask. The mixture was kept at 60°C in a thermostatically controlled water-bath for 3hr.

Aliquots (0.1ml) of the reaction mixture were withdrawn at ½hr intervals. These were blown into test-tubes containing water (1ml) and the material which remained on the walls of the pipettes was rinsed out by repeatedly sucking the solution into the pipette. After the 3hr heating period, the mixture was pink and still contained small amounts of undissolved solid. The diluted samples were heated in a boiling water-bath for 30min and then adjusted to pH 9.5 with lithium hydroxide solution. The precipitates were removed by centrifugation and the supernatant solutions made up to 4ml in graduated test-tubes. Aliquots (0.02ml) of these solutions were applied (4 applications) to the starting line of a paper chromatogram which was developed (Solvent 1) for 21hr (ascending). The paper was dried and the uridine compounds located under the U.V. lamp (see Fig. 6). The spots were cut from the chromatogram (a standard area was taken for each of the components - uridine, U5'P and U2'(3');5'DP) and blanks were cut from areas of the paper which corresponded to the $R_f$ values of the three components, but where no material had been applied at the origin. The small pieces of the chromatogram

were attached to "wicks" of filter paper, which were held between two microscope slides in such a way that the ends dipped into a trough of 0.1N HCl. Eluates were collected for 18hr and then made up to 3ml with 0.1N HCl. The optical density at 260mμ of these solutions was measured and the results are shown in Tables 6a andb. For each $R_f$ value, triplicate blanks were taken and these were read against water (Table 6a). The three blank solutions, corresponding to the $R_f$ value of each compound, were mixed and the other solutions, which contained the spots detected under the U.V. lamp were read against them (Table 6b).

Expt. 13 Preparation of uridine-5'-phosphate.

2':3'-Isopropylideneuridine (2.0g) which had been dried at 100°C in vacuo over $P_2O_5$, was mixed with polyphosphoric acid (10ml) and these were heated together in a stoppered flask held in a thermostatically controlled bath at 60°C for 2hr. Solution of the solid was not complete after this heating period. Water (60ml ) was added and the diluted solution heated on a steam-bath for 30min. Hot, saturated, aqueous baryta was

added to the solution until the pH was approximately 4 and the solution was allowed to cool to 30°C; the pH was then adjusted accurately to pH 6.5, the suspension brought to the boil and filtered while still hot. The volume of the solution (now approximately 400ml) was reduced in the rotary evaporator to 30ml and the pH brought to 7.4 by the addition of a small amount of cold, saturated baryta and the filtrate brought to the boil. The slight precipitate was filtered off and when the filtrate had cooled to room temperature ethanol (100ml) was added. Precipitation was completed by standing the suspension in the refrigerator overnight. The precipitate was collected and washed with ethanol (2 x 25ml), and ether (2 x 25ml). After each washing the solid was collected in the centrifuge. The yield was 2.4g (74% of theory). Paper chromatography (Fig. 6) showed that the product contains a trace of the diphosphate.

The barium salt can be converted to the free acid and further purified as follows. Barium U5'P (1g) was dissolved in water (30ml) with the aid of a trace of acetic acid. This solution was passed through a column

(1.4 x 6.0cm) of Dowex 50 ($H^+$). The column was washed with water until the effluent was no longer acidic and the combined effluents were freeze-dried. The resulting gum was dissolved in warm ethanol (3ml) and this solution was poured into dry ether (50ml) which was stirred mechanically. The white solid which precipitated was collected in the centrifuge and dried *in vacuo*. The yield was 400mg (60% of theory based on Ba U5'P).

Expt. 14. Large scale preparation of U5'P.

In this preparation 10g of dried 2':3'-isopropylideneuridine were reacted with 50ml of polyphosphoric acid. Mechanical stirring ensured that the reactants were thoroughly mixed throughout the heating period. Otherwise reaction conditions and working-up procedure were identical with those employed in Expt. 13. The yield in this preparation was 5.2g of the barium salt (32% of theory).

Expt. 15. Preparaion of ribose-5-pyrophosphate.

Barium ribose-5-phosphate (400mg) was dissolved in water (20ml) with the aid of a trace of acetic acid. The solution was passed slowly through a column (1 x 5cm)

of Dowex 50 ($H^+$) and the column was washed with water until the effluent was no longer acidic. The combined effluents were taken to dryness under reduced pressure; last traces of water were removed by azeotropic distillation with ethanol. The resultant light brown gum was dissolved in absolute alcohol (1.52ml) and added dropwise, with vigorous stirring, to dry ether (20ml). The flocculent white precipitate of R5P, coagulated to a gum from which the ether was decanted. The gum was dissolved in a solution of tri-n-octylamine (0.412g) in dry methanol and the methanol was removed under reduced pressure. Successive solution and evaporation of the tri-n-octylammonium ribose-5-phosphate in anhydrous benzene, toluene and dioxan ensured the removal of traces of water. The residual hard gum was dissolved in anhydrous benzene (5ml) and dioxan (10ml) and to this a solution of dibenzylphosphorochloridate (from 0.406g dibenzylphosphite G.M.20) in anhydrous benzene (5ml) was added. Immediately after this the addition of a solution of tri-n-butylamine (0.243g) in dioxan (10ml) was commenced. This solution was added slowly over a period of 10min, while the mixture was shaken. After 3.5hr at room

temperature, solvents were removed under reduced pressure. The residual, red oil (1.42g) was dissolved in ether (20ml) and precipitated by the addition of petroleum ether (30ml, 60-80% fraction). When the precipitated oil had settled, the ether was decanted and last traces removed by evaporation. The $P^1$-ribose-5-$P^2P^2$-dibenzyl hydrogen pyrophosphate (0.842g) was dissloved in aqueous ethanol (20ml, 80%), palladised charcoal (200mg,10%) was added and the mixture shaken at atmospheric pressure with hydrogen. Uptake of hydrogen (26cc) was complete in 3min. After hydrogenation, the catalyst was filtered off and the solution taken to pH 7.0 with triethylamine. The volume was reduced at the pump to 5ml. Ethanol (25ml) was now added and this was followed by a solution of calcium chloride (0.5g monohydrate) in ethanol (10ml). The precipitate was collected in the centrifuge and washed, first with ethanol (2 x 15ml) and then ether (2 x 15ml). The yield, after drying in vacuo, was 0.212g.

Of this crude calcium salt, 0.12g was dissolved in ether (25ml) with the aid of a few beads of Dowex 50 ($H^+$). The solution was passed through a column (1cm x 6cm) of the same resin and this was washed with water until the effluent was no longer acid. The combined effluents

were adjusted to pH 7.0 by the addition of ammonia, (at this stage the solution was found to contain pentose equivalent to 32.5mg, according to colorimetric analysis). After neutralisation the solution was passed onto a column of Dowex 1 x8 ($Cl^-$, 200-400 mesh, 1cm x 8cm) which was eluted successively with water (100ml), 0.005 N HCl + 0.01N LiCl (200ml) and 0.005N HCl + 0.05N LiCl. The elution was carried out in the cold-room at $4^{\circ}C$, 10ml fractions were collected by means of an automatic syphoning device and aliquots of these (0.2ml or 1ml) were analysed for pentose (G.M. 1). The elution pattern is shown in Fig. 8. Fractions after no. 52 were lost because of a breakdown of the fraction-collector. Fractions 41-52, containing peak II were bulked, adjusted to pH 7.5 with lithium hydroxide and freeze-dried. The solid residue was shaken with ethanolic acetone (100ml of 50%), collected in the centrifuge and washed with ether. The yield, after drying *invacuo*, was 13mg (5.5% of theory). The molar ratio of acid labile to total phosphate was found to be 1:1.85 (G.M. 2).

Expt. 16. Preparation of uridine-5-pyrophosphate.

Barium uridine-5'-phosphate (4g - Expt. 14) was dissolved in water containing a trace of acetic acid and passed slowly through a column (3 x 6cm) of Amberlite IR 120 ($H^+$). The column was washed with water until the effluent was no longer acidic. The combined effluents were taken to dryness under reduced pressure; last traces of water were removed by evaporating several times, solutions of the gum in absolute ethanol. Tri-n-octylamine (3.045g) in ethanol (25ml) was added and after thorough mixing, the solution was evaporated to dryness. The tri-n-octylammonium uridine-5'-phosphate was dissolved successively in dry benzene, toluene and dioxan and the solutions evaporated to dryness under reduced pressure. After this treatment, the glass which resulted was dissolved in dry dioxan (60ml) and benzene (30ml) and a solution of dibenzylphosphorochloridate (from 3.43g dibenzyl phosphite) in dry dioxan (60ml) and benzene (30ml) was added. A solution of tri-n-butylamine (2.405g) in dry dioxan (90ml) and benzene (30ml) was now added dropwise, with shaking over a period of 10min. During the addition precautions

were taken to exclude the atmosphere from the reaction flask. The mixture, which was cloudy, was allowed to stand at room temperature for 3hr during which period aliquots were removed and applied to the starting line of a chromatogram. The chromatogram was afterwards developed in Solvent 3 and is shown in Fig. 9. After 3hr reaction, solvents were removed under reduced pressure and the residue shaken with a mixture of ether (120ml) and petroleum ether (120ml, 80-100° fraction). The precipitated gum was allowed to settle overnight and the supernatant ether was decanted. Traces of solvent were removed at the pump. The gum (7.3g) was dissolved in aqueous ethanol (300ml,70%) palladised charcoal (500mg,10%) was added and the mixture shaken with hydrogen at room temperature and pressure. Uptake of hydrogen (320cc) was completed in 30min and the ethanolic solution was filtered from the catalyst. After adjusting to pH 7.4 by addition of triethylamine, the solution was again filtered and a solution of calcium chloride (6g, monohydrate) in aqueous ethanol (30ml,70%) added. The precipitate was collected in the centrifuge and washed with ethanol (2 x 60ml) and ether (2 x 60ml).

The yield, after drying *in vacuo*, was 3.4g.

An attempt to purify this crude product by calcium salt fractionation described by Michelson (170) was carried out as follows. The crude mixture of calcium salts (200mg) was suspended in water (1.5ml) and dilute hydrochloric acid was added carfully, with stirring, to pH 4.2. Undissolved material was centrifuged down and discarded. The supernatant solution was taken to pH 2.5 with hydrochloric acid and to this solution ethanol (6ml) was added. The precipitate was collected by centrifugation and washed with ethanol (2 x 5ml) and ether (2 x 5ml). The product (108mg) was shown by paper chromatography in Solvents 3 and 4 to contain small amounts of U5'P and larger amounts of an unidentified substance with $R_f$ less than UDP.

The remainder of the calcium salts (3.2g) was fractionated by ion exchange chromatography. A solution of the calcium salts in hydrochloric acid (120ml, N/1000) was passed through a column (3 x 6cm) of Amberlite IR 120 ($H^+$) and this was washed with water

until the effluent was no longer acidic. The combined effluents ($TOD_{260}$ = 50,500) were adjusted to pH 6.8 with ammonia and applied to a column (6 x 10cm) of Dowex 1x8 (200-400 mesh, $Cl^-$ form). After washing the column with water (1,200ml) until the $O.D._{260}$ had fallen below 0.1, the elution was continued with 0.003N HCl + 0.04N LiCl (7,500ml), 0.03N HCl + 0.1N LiCl (12,500ml), 0.008N HCl + 0.1N LiCl (4,500ml), 0.01N HCl + 0.1N LiCl (5,000 ml), 0.01N HCl + 0.2N LiCl (9,000 ml) and finally 2N HCl. Elution was carried out in the cold-room at 4°C. At first, 25ml fractions were collected, but the fraction size was increased to 75ml after 20l. of effluent had passed. Each fourth fraction was examined for uridine compounds by measuring the $O.D._{260}$. The elution pattern is shown in Fig. 10. Fractions containing peaks I, II and III respectively were bulked and the three solutions obtained were adjusted to pH 7.0 by addition of lithium hydroxide. Thereafter, the three were treated separately in an identical manner. The solution was evaporated to dryness in the rotary evaporator. The last traces of water, which were difficult to evaporate, were removed by azeotropic distillation with ethanol. The resulting

cake was shaken with anhydrous methanol (50ml were used for peak II) and the viscous solution was centrifuged. After shaking with a small amount of dry methanol, the precipitate was washed onto a sintered glass filter where washing with methanol was continued until a negative test for chloride was obtained. Successive washings with acetone and ether removed the methanol and the precipitate was dried *in vacuo*. The yields were, peak I - 100mg, peak II - 1.07g, peak III - 197mg.

paper chromatography in solvents 3 and 4 (Fig. 11) showed that peak I is identical with U5'P and peak II with UDP. Analysis of peak II gave a molar ratio of uracil:acid labile phosphate:total phosphate =1:1.23:2.3.

Expt. 17. *Preparation of 4,5-dihydrouracilriboside-5'-pyrophosphate.*

Uridine-5'-pyrophosphate (10mg sodium salt) was dissolved in ammonium acetate buffer (10ml, 0.1M, pH4) and shaken with 5%rhodium on alumina (10mg) in an atmosphere of hydrogen. At intervals aliquots (0.02ml) were withdrawn, diluted with water (1ml) and the $O.D._{260}$

measured. After 18hr, when the $O.D._{260}$ had fallen to zero, catalyst was filtered off and the solution was adjusted to pH 8 by the addition of caustic soda. This solution was stored frozen at -18°C.

Experiments for Section II.

Expt. 18. Preparation of protein extract from E. coli by ultrasonication.

The wet cell paste (14g grown as in G.M. 10) was suspended in glycylglycine buffer (14ml, ph 7.4, 0.05M) in a wide test-tube, cooled in an ice-water bath. The tip of the M.S.E. 9Kc ultrasonic disintegrator was inserted just below the surface of the suspension and the pitch of the oscillator adjusted until maximum "resonance" in the liquid was obtained (this was judged by observing the agitation of the cells). Samples were withdrawn at 5 minute intervals and examined under the microscope. It was clear that some disruption did occur, but a high proportion of whole cells remains after 30min operation.

Expt. 19. Treatment of E. coli with lysozyme in the presence of EDTA.

The solution of lysozyme/EDTA was prepared by mixing equal volumes of solutions (1) Tris (100mg) made to ph 8.0 with HCl and diluted to 100ml with water and (2) Lysozyme (26mg) + EDTA (106mg, Na salt) made to

pH 8.0 with KOH and diluted to 100ml with water. E. coli (40mg wet cell paste G.M. 10) were suspended in 4ml of distilled water and mixed with 4 ml of the lysozyme solution in a colorimeter tube. The optical density at 660mμ was read at frequent intervals (Fig. 14).

Expt. 20. Preparation of E. coli extracts by alumina grinding.

The cell paste (prepared according to G.M. 10) was ground with double its weight of polishing alumina (Aluminium Company of America A 305) in a chilled pestle and mortar. The mixture became tacky after approximately 10 min grinding. Grinding was continued for a total of 25 min and the paste was then thinned by small additions of glycylglycine buffer (pH 7.5, 0.05M) (a total of 300ml of buffer was used for each 100g of cell paste). After centrifuging (20 min at 6,000 r.p.m.) the supernatant solution was decanted and the solid debris was re-extracted with the buffer (200ml for each 100g of original cell paste). Centrifuging was repeated, the supernatant solutions from the two extractions were combined and the debris discarded. The combined protein solutions

were re-centrifuged (30min at 16,000 r.p.m.) and stored frozen in 25ml portions until required.

Ext. I was prepared from 93g of wet cell paste and Ext. II from 38g.

Expt. 21. Demonstration of polynucleotide phosphorylase activity in crude E. coli extract.

A solution of ADP (25mg, Na salt - Sigma Chemical Co.) in Tris buffer (pH 8.2, 1.5ml, 0.2M) containing $MgCl_2$ (0.01M) was mixed with the crude extract (0.4ml, Ext. I) and the mixture incubated at 37°C in a thermostatically controlled water-bath. Aliquots (0.1ml) were withdrawn at intervals (½hr) and the reaction stopped by heating in a boiling water-bath (2min). This last step was carried out in conical centrifuge tubes and after removing the precipitate by centrifugation, the supernatant solution was applied to the origin of a chromatogram (Whatman No. 1 filter paper). The chromatogram was developed in Solvent 4 (Fig. 16).

Expt. 22. Time course of the reaction of polynucleotide phosphorylase (alumina C$\gamma$ eluate) with two commercial samples of ADP.

Assays were carried out according to G.M. 11. The ADP stock solution was prepared using two different samples of ADP. (1) from Biochemica Boehringer AG (2) from Sigma Chemical Co.; the same concentration of ADP was used in each case. Six digests for each ADP sample were set up and 0.1ml of alumina C$\gamma$ eluate (Expt. 29d) was added to each. The reactions were stopped by the addition of perchloric acid after 0, 3.5, 7, 15, 33, and 65 min respectively and phosphate determined. The results are illustrated in Figs. 17a and b.

Expt. 23. Likely contaminants of ADP tested as substrate for phosphatase activity with alumina C$\gamma$ eluate.

A list of phosphate esters thought to be possible contaminants of commercial ADP is given in Table 7, together with some others. Solutions (10mg per ml) of the sodium salt of these compounds were made in the buffer used in the assay procedure (G.M. 11). Aliqots

(0.1ml) of these solutions were mixed with an equal volume of the enzyme solution (alumina Cγ eluate, Expt. 29d) and thereafter treated as the digests in the assay of polynucleotide phosphorylase. A digest period of 5min was used in each case. The phosphate release from each substrate is shown in Table 7.

Expt. 24. Separation of ADP from the contaminant by adsorption on charcoal.

A solution of ADP (10mg, Biochemica Boehringer AG) in water (1ml) was treated with acid washed Norit A until the optical density at 260mμ of the supernatant solution was negligible. 0.1ml of this solution was mixed with an equal volume of the standard assay buffer (G.M. 11) and 0.2ml of enzyme solution (alumina Cγ eluate, Expt. 29d) after 5min digestion, phosphate was determined according to the usual procedure (G.M. 11and 2). The phosphate release was 6 micrograms. In a similar experiment with untreated ADP, phosphate release was 8.5 micrograms.

Expt. 25. Treatment of E. coli extract with manganese chloride and protamine.

Freshly prepared manganese chloride solution (0.2ml, 1M) was added slowly to crude E. coli extract (Ext. I, 4ml) which was kept cool by an ice-water bath and stirred mechanically. Stirring was continued for 30min after which the flesh coloured precipitate was removed by centrifugation (20min,20,000g). The light yellow supernatant solution was dialysed overnight, in the cold against potassium chloride solution (1l.,0.9%). To the dialysate (2ml) was added slowly, with mechanical stirring a solution of protamine sulphate (0.2ml,1% L. Light, Ltd.). The precipitate was removed by centrifugation. For enzyme assays (G.M. 11) aliquots (0.1ml) of the dialysed manganese supernatant after dialysis and the protamine supernatantsolution were used without further treatment. Addition of more protamine sulphate (0.2ml) to the protamine supernatant solution caused no visible precipitation.

Expt. 26. Treatment of crude E. coli extract with bentonite.

Crude extract II was dialysed for 12hr against potassium chloride solution (100vols, 0.9%. 4°C).

Bentonite suspension was prepared according to G.M. 13 . In six conical centrifuge tubes were mixed 0.25ml dialysed Ext. II with a)0.00ml b)0.05ml c)0.10ml d)0.15ml e)0.20ml f)0.25ml of bentonite suspension and the volume in each tube was made up to 0.50ml by the addition of water. After 15min at 0°C the tubes were centrifuged and samples of the supernatant solution withdrawn for assays (G.M. 11) and protein estimation (G.M. 12a). The results (Table 9) are illustated in Fig. 18.

Expt. 27 Treatment of crude E. coli extract with charcoal.

The procedure in this experiment was similar to that used in Expt. 26. The volumes of charcoal suspension (G.M. 15) were a)0.00ml b)0.025ml c)0.05ml d)0.10ml e)0.15ml and the volume in each tube was made up to 0.4ml with water. The supernatant solutions, after centrifuging down the charcoal, were assayed for polynucleotide phosphorylase (G.M. 11) and protein (G.M. 12b) (Table 10).

Expt. 29. Treatment of crude E. coli extract with calcium phosphate gel.

Calcium phosphate gel (G.M. ,4ml) was packedinto a loose pellet in the centrifuge (2min at 2,000 r.p.m.) and the supernatant solution decanted. The crude extract (10ml, Ext. I) was added and the gel dispersed in the protein by high speed mechanical stirring. After a period of stirring (15min), during which care was taken to avoid frothing, the gel was removed in the centrifuge (15min at 6,000r.p.m.). The supernatant solution (after removing sufficient for activity estimation) was treated in a similar manner with a further amount of gel. The process was repeated so that, in all, 7 additions of gel (4ml each) had been made. The results of activity tests (G.M. 11) are shown in Table 19.

| Protein soln. | Pi release in 15 min |
|---|---|
| Crude ext. | 10.5 |
| gel sup. 1 | 11.0 |
| " 2 | 9.0 |
| " 3 | 9.0 |
| " 4 | 8.0 |
| " 5 | 7.0 |
| " 6 | 6.0 |
| " 7 | 5.5 |

Expt. 29a. Fractionation of polynucleotide phosphorylase on alumina C$\gamma$ .

Alumina C$\gamma$ (1ml, 20mg dry weight per ml, G.M.17) was packed into a pellet by centrifuging. Crude extract (8.5ml, Ext. 1) was added and thoroughly mixed with the gel by high speed mechanical stirring. This process was repeated with further amounts of gel (3 x 2ml) After each gel treatment and centrifugation, solution was removed for activity determination: these are shown in Table 11. The last three portions of gel were combined and suspended for 30min in water (3ml). The gel was centrifuged down and stepwise elution was continued in the same manner as the water wash, with buffer solutions. The nature and concentration and volume of the buffers, the time of contact with the gel, and the activity and protein concentration of the eluates are given in Table 11.

Expts. 29b,c and d. Fractionation of polynucleotide phosphorylase on alumina C$\gamma$ .

These experiments differed from Expt. 29a only in the quantities of crude Ext. 1 used, the amounts of alumina C$\gamma$ added, and the buffers used to elute the gel.

These details are given in Tables 12-14.

For use in other experiments, fractions 9-14 eluted from alumina C$\gamma$ by sodium bicarbonate solution in Expt. 29d (Table 14) were dialysed against potassium chloride solution (100vols, 0.9%). The dialysed solution is referred to as dialysed alumina eluate.

Expt. 30. Improved method for fractionation of polynucleotide phosphorylase on alumina C$\gamma$.

The crude E. coli extract (Ext. II, 20ml) was stirred mechanically while manganese chloride solution (1.0ml, 1.0M) was added slowly. The precipitate was centrifuged down (20min,20,000g) and the supernatant solution was dialysed overnight against Tris buffer (2l. 0.005M, pH 7.4) in the cold-room at $4^{o}$C. The cloudy solution was clarified by centrifugation and treated with alumina C$\gamma$ gel (0.5ml) in the manner described in Expt. 29a. After removal of the gel in the centrifuge, the supernatant was treated with additional portions (0.5ml, 0.5ml, 1ml, 1ml). The results of assaying the supernatant solutions obtained from these

additions of gel are shown in Table 15 and Fig. 20a. The last two portions of the gel were combined and washed with water (5ml) into the apparatus designed for the continuous elution of gels (Fig. 19). An automatic fraction collector was set up to take 5ml fractions and a reservoir containing saturated sodium bicarbonate was attached to the inlet arm. The gel was eluted, with slow stirring, at a rate of 1.5 fractions per day. Each fraction was assayed for activity (G.M. 11), and protein (G.M. 12b) and the optical densities at 260mμ and 280mμ were measured. The results are given in Table 15 and the course of elution is shown in Fig. 20b. Eluted fractions 2,3 and 4 were combined for further experiments.

Expt. 31. Removal of ribonuclease from alumina eluate by adsorption on bentonite.

Combined fractions 2, 3 and 4 from Expt. 30 were used in this experiment. Aliquots (0.5ml) of this solution were shaken with varying amounts (0.0 - 0.1ml) of bentonite suspension (G.M. 13,) in conical centrifuge tubes. The volume of each mixture was made up to 0.6ml with water and the tubes

were centrifuged. The results of assays and protein estimations on the supernatant solutions are shown in Table 16. Ribonuclease activity could not be detected (G.M. 14) after the first addition of bentonite.

Expt. 32. Further fractionation of alumina eluate on DEAE cellulose: preliminary trial.

In this and the subsequent large scale experiments dialysed alumina eluate from Expt. 29d was used. The DEAE cellulose was prepared in the chloride form as described in G.M. 21. The dialysed protein solution (3ml) was applied to a small column (3 x 10mm) of the ion-exchange cellulose at an unrestricted flow-rate. Solutions of potassium chloride (in 0.5 ml portions) were used to elute the column. Seven fractions were used in all. The concentration of the eluting agents and the results of assays are given in Table
Protein estimations, using the Lowry reagents (G.M. ) were inconclusive because the KCl formed a precipitate with the alkali.

Table 20

Stepwise elution of polynucleotide phosphorylase from DEAE-cellulose by potassium chloride.

| Fraction no. | concn. | Pi release in 30 min. |
|---|---|---|
| 1 | 0.1M | 0.0 |
| 2 | 0.1 | 0.0 |
| 3 | 0.2 | 3.0 |
| 4 | 0.2 | 5.0 |
| 5 | 0.2 | 8.0 |
| 6 | 0.2 | 3.0 |
| 7 | 0.2 | 1.0 |

Expt. 33. Fractionation of alumina eluate on DEAE cellulose, using gradient elution.

A column of DEAE-cellulose (8 x 100mm) was prepared (G.M. 21) and dialysed alumina eluate (Expt. 29d) was applied over 18hr. After a preliminary wash with sodium chloride (5ml, 0.01M) a linear concentration gradient was used to elute the column. The flow-rate was 2ml/hr. Fractions (2ml) were collected automatically. The gradient of sodium chloride (0.01M - 0.5M) was contained in a total volume of 200ml. The effluent was tested for phosphate release with ADP (G.M. 11) and for

protein by measuring O.D. at 280mμ. The elution pattern is shown in Fig. 21.

Expt. 34. Further fractionation of alumina eluate on alumina C .

Treatment with gel was identical to that used previously (Expt. 29). Dialysed alumina eluate (4ml) was treated successively with two portions of alumina C (0.1ml and 0.075ml). After centrifugation the gel fractions were eluted with saturated sodium bicarbonate solution (0.3ml each fraction). Results of polynucleotide phosphorylase assays and protein estimation are shown below.

Table 21.

Re-fractionation of alumina eluate on alumina C .

| Protein soln. | Vol.ml. | Pi release in 30 min. | Protein |
|---|---|---|---|
| dial. Al elute | 4 | 10 | 0.5 |
| 1st Al sup. | .1 | 4 | 0.13 |
| 2nd " | .075 | 0 | 0.12 |
| combined al eluted | .3 | 18 | 0.2 |

Expt. 35. Fractionation of alumina eluate on calcium phosphate gel.

The protein solution used in this experiment was dialysed alumina eluate (from Expt. 29d). Calcium phosphate gel (G.M. 16) was added in 3 portions (0.2ml, 0.15ml, 0.15ml) successively and these were removed by centrifugation. The gel fractions were combined and eluted with saturated sodium bicarbonate. Results of activity and protein estimations are shown in Table 22.

Table 22

Fractionation of alumina eluate on calcium phosphate gel.

| Protein soln. | Vol. gel (ml) | Vol. soln. (ml) | Pi release in 30 min. | Protein (mg/ml) |
|---|---|---|---|---|
| dialysed Al eluate | 0 | 4 | 10 | .5 |
| $(Ca)_3(PO_4)_2$ sup 1 | .2 | 4 | 7 | .2 |
| " " 2 | .15 | 4 | 5 | |
| " " 3 | .15 | 4 | 2 | |
| 1 $NaHCO_3$ | | .3 | 10 | .18 |
| 2 " | | .3 | 32 | .18 |
| 3 " | | .3 | 17 | .10 |
| 4 " | | .3 | 15 | .10 |

Expt. 36. Attempted synthesis of ribose phosphate polymer from ribofuranose-5-pyrophosphate.

Ribofuranose-5-pyrophosphate (7mg, Li salt) was dissolved in the standard buffer used to prepare the assay mixture (G.M. 11, 0.5ml) and protein solution (alumina eluate from Expt. 29d, 0.5ml) was added. The mixture was incubated at 37°C in a stoppered test-tube under toluene. Aliquots (0.05ml) were withdrawn at intervals for determination of inorganic phosphate (G.M. 2). After 48hr incubation, no detectable inorganic phosphate had been released.

Expt. 37. Attempted synthesis of polydihydrouridylic acid from 4:5-dihydrouracilriboside-5'-pyrophosphate.

The solution of dihydrouracilriboside-5'-pyrophosphate, prepared in Expt. 17 (1ml) was adjusted to pH 8.2 with caustic soda and magnesium chloride was added (to a final concentration of 0.01M). Enzyme solution (0.5ml, alumina eluate from Expt. 29d) was added and the mixture incubated at 37°C. Aliquots (0.1ml) were removed for determination of inorganic phosphate

(G.M. 2). After 24hr incubation no detectable inorganic phosphate had been released.

Expt. 38. The synthesis of polyU by the action of polynucleotide phosphorylase on UDP (1).

The lithium UDP prepared in Expt. 16 (50mg) was converted to the sodium salt (G.M. 22) and freeze-dried. The solid was dissolved in Tris buffer (1ml, pH 8.0, 0.2M) containing magnesium chloride (10mg) and the solution mixed with enzyme solution(bentonite treated alumina eluate from Expt. 29d, 4ml). The mixture was incubated at 37°C. Aliquots (0.01ml) were withdrawn at intervals for inorganic phosphate determination. After 4 hr incubation the phosphate release corresponded to 30% approximately of the total acid labile phosphate. The digest mixture (after 36hr at 37°C) was applied to a column of Sephadex G25 (2 x 25cm, G.M. 18) and eluted with water. Fractions (1ml) were collected and the optical density at 260mu measured. The elution pattern is shown in Fig. 23a. Fractions 20-29 (11ml) and 30-53(30ml) were combined and the optical density of the two solutions determined (5.5 O.D. units and 8.4 O.D. units respectively).

Thus the T.O.D.$_{260}$ of the first peak is 20% approximately of the combined peaks.

Expt. 39. The synthesis of polyU by the action of polynucleotide phosphorylase on UDP (2).

Lithium UDP (prepared in Expt. 16, 310mg) was dissolved in the $Mg^{++}$/Tris buffer used for making the assay mixture (G.M. 11, 30ml) and mixed with enzyme solution (bentonite treated alumina eluate from Expt 30, 7.5ml). After 9hr incubation the mixture was applied to a column of Sephadex G25 (3.5 x 12 cm) which was then eluted with sodium chloride solution (0.2M). Fractions (5-6ml) were collected at a flow-rate of 200ml/hr. The elution pattern is shown in Fig.23c. Fractions 23-35 (80ml, T.O.D.$_{260}$ = 1,550) and 39-63 (150ml, T.O.D.$_{260}$ = 4,,020) were combined. According to the T.O.D.$_{260}$ of these solutions the yield of polymer was 28%. The solution containing the first peak was treated with the standard bentonite suspension(2ml, G.M. 13), centrifuged and dialysed for four days against three changes of distilled water (7l.). The yield of white solid obtained by freeze-drying the dialysate was 60mg. It was stored

at -18°C in a sealed bottle.

Expt. 40. Synthesis of polyuridylic acid by the action of diphenylphosphorochloridate on uridylic acid.

Commercial uridylic acid (1.0g dried 18hr over $P_2O_5$ in vacuo) was dissolved, with tri-n-octylamine (1.11g) in anhydrous methanol. The solution was evaporated to dryness under reduced pressure and the residual solid foam was successively dried from solutions in dry dioxan, toluene and benzene. To a solution of the anhydrous solid in dioxan (13ml) was added diphenylphosphorochloridate (0.97ml) and tri-n-butylamine (1.94ml). After 2hr at room temperature, the mixture was treated with further similar quantities of diphenylphosphorochloridate and tri-n-butylamine and the reaction allowed to proceed for 3hr longer. Solvents were removed under reduced pressure to produce a light brown oil, from which the product was precipitated by the addition of ether (100ml). The oil was dissolved in water (15ml) adjusted to pH7 by the addition of ammonia and extracted 3 x with ether (2 vols). The aqueous layer was freeze-dried (1.4g). A portion of this solid (1.21g) was dissolved

in ammonium bicarbonate buffer (2ml, 0.2M) and clarified by centrifugation. The precipitate was extracted with the buffer (0.5ml) and the supernatant solutions combined. This viscous solution was applied to a column of Sephadex G25 (1.5 x 62.5cm) which was subsequently eluted with the ammonium bicarbonate buffer. Fractions (10ml) were collected and aliquots of each applied to the starting line of a chromatogram which was developed in Solvent 3. The effluent was bulked into three fractions as described in Section II and these were separately freeze-dried until they no longer smelled of ammonia. The yields from the first second and third fractions, were 340mg, 305mg and 315mg respectively.

Experiments for Section III.

Experiment 41. Determination of optimum pH for reaction of hydrazine with poly U.

Solutions of hydrazine(2M,2ml), adjusted to pH2-12 by the addition of sulphuric acid mixed with an equal volume of a solution of poly U(chem.synth. Sephadex fraction I),(1ml containing 0.06mg). The optical density at 260mu was read immediately after mixing and again after 1.5hr. at room temperature. The blank solutions used in spectrophotometric determinations were prepared by mixing the appropriate hydrazine solution with an equal volume of water. Results are shown in Fig.25

Expts.42,43,44. Preliminary experiments with three methods for uracil destruction on poly U; products isolated on Sephadex.

Each of three methods was applied. to chem, synth. polyU (Sephadex fraction I) as described below. After treatment the solution,in each case,was applied to a column(0.7x50cm.) of Sephadex G25 which had been equilibrated with sodium chloride solution(0.2M,G.M.18). The column was eluted using the same salt solution and fractions(10drops)

were collected. After measuring the optical density at 260mμ of each fraction, aliquots(0.1ml) were taken for estimation of pentose(G.M.1,4) and reducing power(G.M.3,4) R Results are illustrated in Figs26-28, in these the reducing power estimations are omitted as this was only shown by fractions containing hydrazine or hydroxylamine. None was shown by peaks containing pentose.

42. Reaction with hydrazine at pH 9.

Chem synth. polyU(1mg., Sephadex fractionI), was dissolved in aqeous hydrazonium chloride(1,0ml,1OM,pH9.0) and the solution held at 37°C in a thermostatically controlled water-bath. Aliquots(0.01ml) were withdrawn at intervals and diluted with water(1.0ml). The optical density of these solutions at 260mu was measured, and when this has reached a minimum(approx.2hr.) the remainder of the reaction mixture was applied to the column of Sephadex G25 as described above.

43. Reaction with hydroxylamine at pH 10.

Chem.synth. polyU(1mg Sephadex fraction1)was dissolved in aqeous hydroxylammonium chloride(1.0ml,1OM,pH10.0)and the solution was kept at room temperature. Aliquots(0.01ml)

were withdrawn at intervals and diluted with water(1.0ml). The optical density at 260mμ of these solutions was measured and when this had reached a minimum(approx 2.5hr) the remainder of the reaction mixture was applied to the column of Sephadex G25 as described above. After the colorimetric estimation had been carried out, the eluted portions were shaken with a solution of benzaldehyde in ether (2x1volume.20%w/v). After each addition the solutions were shaken vigorously and allowed to stand at room temperature for 2hr. in one treatment, for 18hr. in the other. Excess benzaldehyde and the benzaldoxime were removed by washing with ether (3x3volumes) (The addition and removal of solutions to the fractions, which were contained in small fraction-collection tubes, were made using a dropping pipette) After the final ether wash, the colorimetric determinations were repeated

44. Hydrolysis following catalytic hydrogenation.

Chem synth polyU (1mg., Sephadex fraction.) was dissolved in water (0.5 ml) and the solution was adjusted to pH 3.0 by the addition of hydrochloric acid. A small amount of catalyst (5% rhodium on alumina) was added and the mixture was shaken

with hydrogen at room temperature and atmospheric pressure At intervals the shaking was stopped, the catalyst was centrifuged down and an aliquot (0.01ml) carefully removed from the supernatant solution. The optical density of the sample was measured after dilution with water. When this had fallen to zero, the catalyst was removed by centrifugation and the solution was made alkaline by the addition of sodium hydroxide solution (0.1vol, 1.0N) Hydrolysis was allowed to proceed for 1hr. at room temperature and then the solution was acidified by the addition of hydrochloric acid (0.2volumes, 1.0N) .After 24hr. at room temperature, the solution was applied to the Sephadex column as described above.

Expt.45. Reaction of uridine-5'-phosphate with hydrazine hydrate.

Uridine-5'-phosphate (40mg, freeacid) was dissolved in hydrazine hydrate (0.4ml) and held at 70°C for 10min. The optical density at 260mμ of a sample (0.01ml) diluted to 1.0ml. with water, was zero after reaction. The reaction mixture was diluted and poured through a column of Amberlite IR 120 ($H^+$, 2x20cm) and the column was washed with water until the effluent was no longer acidic. The combined effluents were taken to pH 6.3 by the addition of lithium hydroxide solution

and freeze-dried. To remove all traces of the hydrazine, the lithium salt was dissolved in dry methanol.(7ml) and reprecipitated with dry acetone (150ml). The precipitate was centrifuged,washed with ether,and dried (20mg theory=32mg assuming conversion to R5P). For paper chromatography (G.M.5 ,solvents2,6) the lithium salts were converted to ammonium salts(G.M.22 ).

Expt. 46. Reaction of uridine-2'(3')-phosphate with hydrazine hydrate.

Reaction was carried out exactly as described for uridine-5'-phosphate using the mixed 2'- and 3'- isomers (commercialU2'(3')P,L.Light and Co.). After reaction the hydrazine was removed by treatment with AmberliteIR120 (H+) and the solution was then stored overnight in an evacuated dessicator containing sodium hydroxide and phosphorus pentoxide. After conversion to ammonium salts the solutions of the phosphate esters were applied to filter paper (Whatman No.1).

Expt.47. Hydrogenation of uridine-5'-phosphate followed by hydrolysis.

Uridine-5'-phosphate(40mg,free acid) was dissolved in water

(17.5ml,pH=2.5). Rhodium on alumina (20mg) was added and the mixture shaken with hydrogen at room temperature and atmospheric pressure. When the optical density at 260mμ had fallen to zero (approx.1.5hr) the catalyst was filtered off. The filtrate was made 0.1N with respect to sodium hydroxide and allowed to stand at room temperature for one hour. The solution was taken to pH 7.0 by the addition of sodium hydrochloric acid and shaken with excess Amberlite IR120 (H+) cation exchange resin at room temperature for 24hrs. The resin was removed in the centrifuge,washed with water and the combined supernatant solutions were taken to pH 6.3 by the addition of lithium hydroxide solution. After freeze-drying,the lithium salts were triturated with anhydrous methanol (5ml) ,dry acetone (100ml) was added and precipitation completed by standing the suspension in the refrigerator for 2hr. The precipitate was centrifuged down ,washed with 50% acetone/ ether (20ml) and dried. For paper chromatography, the lithium salts were converted to ammonium salts (G.M.22).

Expt.48. The action of alkali on ribose-5-phosphate.

Barium ribose-5-phosphate(10mg) was converted to the sodium salt(G.M.22) and the solution made up to 2ml. Of this solution 0.25ml. was taken and diluted to 15ml with 0.0 N sodium hydroxide solution. The alkaline solution was placed in a boiling water-bath and samples (0.5ml) were withdrawn at intervals and tested for pentose and inorganic phosphate (G.M. 1 and 2). Results showed that the pentose is 95% destroyed in 15min. and totally destroyed in 40min. Phosphate release is slower.

Expt. 49. Treatment of polyU with hydrazinium chloride at pH 9.0; product isolated on ECTEOLA cellulose.

Chem. synth polyU (50mg, Sephadex fraction I) was dissolved in aqueous hydrazine (1ml, 10M, adjusted to pH9.0 with HCl) and the solution was incubated at 37°C until the optical density at 260mu had fallen to a minimum (approx. 3hr.) The solution was diluted with water (250ml) and passed through a column of Amberlite IR 120 (H+). The column was washed with water (750ml) and the combined effluents were taken to pH 7.0 by the addition of sodium hydroxide solution. This solution was applied to a column of ECTEOLA-cellulose (containing approx. 5ml of cellulose) which was subsequently eluted with a) water (250ml), b) 0.05M HCl (250ml), c) 0.25M HCl (250ml). Fractions (10ml) were collected automatically after addition of the 0.05M LiCl, and tested for pentose content (G.M.1,4). Fractions 29-35, containing the second pentose peak, were combined and freeze-dried. The solid was taken up in anhydrous methanol (4ml) and dry methanol (100ml) was added. The precipitate was centrifuged down, washed with acetone and ether (100ml) and and dried. Yield, 22mg = approx. 60% of theory (passed on ribose-phosphate polymer) Nitrogen = 6.43%.

For determination of reducing pentose

(G.M.23) a solution containing 1.04mg in Tri s buffer (2ml, pH7.5) was prepared. Aliquots were treated as described in G.M.23.

Expt.50. Synthesis of polydihydrouridylic acid.

Chem. synth. polyU (240mg, Sephadex fraction I) was dissolved in water and converted to the acidic form (G.M.22). The solution was diluted to 20ml with water and 5% rhodium on alumina (250mg added).

The mixture was shaken with hydrogen at room temperature and atmospheric pressure until the optical density at 260mμ had fallen to zero (approx.24hrs.) Catalyst was filtered off and the filtrate freeze-dried. The yield of polydihydrouridylic acid was 185mg (75% of theory)

Expt.51. Treatment of polydihydrourydilic acid with alkali and acid to remove dihydrouracil residue.

Polydihydrouridylic acid (60mg, synthesised in Expt. 50) was dissolved in sodium hydroxide solution (10ml, 0.1N). Aliquots (0.01ml) were withdrawn at intervals and run into sodium hydroxide solution (0.2ml, 0.1N). The optical density at 235mμ of these solutions was measured and when this

had fallen to zero(60min) the alkali was removed by addition of Amberlite IR 120 to pH 4.

After filtering off the resin, the solution was made 0. N with respect to hydrochloric and kept at room temperature for 24hr. The solution was then adjusted to pH6.5 by addition of sodium hydroxide solution and diluted to 500ml with water. Chromatography and isolation of the lithium salt on ECTEOLA-cellulose were carried out exactly as described for hydrazine treatment polyU (Expt.49.)

The yield was 12.6mg (28%theory)
Nitrogen=1.94%.

For determination of reducing pentose (G.M.23) a solution containing 0.96mg in Tris buffer (2ml, pH 7.5) was prepared. Aliquots taken from this solution were prepared. as described for R5P in G.M.23.

Expt. 52. Preparation of polyhydrouridylic acid from polyUridylic acid synthesised by polynucleotide phosphorylase.

Polyuridylic acid synthesised in Expt.38

(3160.D. units;equivalent to approx. 10mg polyU) was used. The solution obtained from the Sephadex column (11ml) was adjusted to pH 3.0 by the addition of hydrochloric acid, 10mg of 5% rhodium on alumina was added and the mixture shaken with hydrogen until the optical density had fallen to zero (approx 10hr.). In alkali (0.1N sodium hydroxide) this reduced polymer showed the specific absorption at 235mμ characteristic of dihydrouracil.derivatives, which was rapidly reduced by standing in alkali.

Expt.53. Attempts to hydrogenate polyuridylic acid synthesised in Expt.39.

Polyuridilyc acid (3mg sodium salt from Expt.39) was converted to the free acid (G.M.22) and the solution made up to 1.5ml. Catalyst (2mg, 5% rhodium on alumina) was added and the mixture shaken with hydrogen at room temperature and atmospheric pressure. Aliquots (0.02ml) were withdrawn and dilution to 1ml for determination of optical density at 260mμ. No reduction was observed after 8hr. hydrogenation.

The catalyst was removed, the solution adjusted to pH3 by the addition of hydrochloric acid and fresh

catalyst (10mg) was added. Hydrogenation was continued for 18hrs. This and a subsequent similar treatment, did cause a drop optical density at 260mμ. However, total phosphate estimation (G.M.2) on the solution showed that material had been removed from solution possibly by adsorption to catalyst or precipitation as aluminium salt.

A further amount of the polyuridylic acid (3mg) was dissolved in water (3ml) and standard bentonite suspension (0.1ml., G.M.13) was added. After mixing, the bentonite was centrifuged down and the supernatant solution treated with Amberlite IR 120 (H+) as described above. An attempt to hydrogenate the acidic solution over 5% rhodium on alumina (3mg) did not cause any fall in the optical density at 260mμ. Catalyst was removed and the solution shaken with chloroform (2x2ml) which was subsequently removed by withdrawing into a pipette. Traces of chloroform were evaporated under reduced pressure. Hydrogenation was repeated as above. Again no fall in optical density at 260mμ was observed.

After removing the catalyst the solution was applied to the column of SephadexG25 (2.5x25cm) and eluted with water. The peak containing the polyU was isolated, catalyst was added and hydrogenation repeated as above. No fall in optical density at 260mμ was observed

after shaking for 24hrs with hydrogen at room temprtature and atmospheric pressure.

The solution was again treated with Amberlite IR120 (H+) and fresh catalyst (3mg) added. The mixture was shaken with hydrogen at 5 atmospheres. No reduction in optical density at 260mμ was observed after 3days.

Expt. 54. Comparison of the rates of hydrolysis of polyuridylic acid and polydihydrouridylic acid by pancreatic ribonuclease.

The two substrates (poly-uridylic acid, Sephadex fraction I and polydihydrouridylic acid, from Expt. 50) were dissolved in ammonium acetate solution (1.0M) containing magnesium chloride (1.0N) to give a concentration of 1.0 mg/ml. Enzyme solution was prepared by dissolving crystalline pancreatic ribonuclease in the same buffer, containing 1mg per ml of bovine serum albumin.. Digests were prepared by mixing substrate solution (0.3 ml) with enzyme solution (0.02 ml). A drop of toluene was added to each and they were then allowed to stand at room temperature. Aliquots (0.02 ml) withdrawn at intervals, were diluted to 1.0 ml with water and fractionated on ECTEOLA-cellulose as described in G.M.19. For polyU, the optical density of the eluted fractions was

measured at 260 mμ. The dihydropolyuridylic acid fractions were tested for pentose according to G.M.L. The total amount of substrate in the digest mixture was estimated by diluting an aliquot (0.02 ml) to 3 ml and measuring the optical density at 260 mu or the pentose content. In Table ? , % reaction is the % material which is eluted from the ECTEOLA-cellulose by 0.05m lithium chloride.

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